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Developing an On-line Extraction-Reaction Process for Lipids Using Supercritical Carbon Dioxide

By

Karamatollah Rezaei

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of *Doctor of Philosophy*

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Fall 1999

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ABSTRACT

Lipase-catalyzed hydrolysis of canola oil in supercritical CO_2 ($SCCO_2$) was used as a model reaction to develop an on-line extraction-reaction system to extract oil from oilseeds and to convert them to high-value products in a supercritical fluid (SCF). The optimum reaction conditions (24 MPa and 35°C) were obtained by investigating the effect of pressure, temperature and CO_2 flow rate on the continuous hydrolysis of a commercially refined canola oil. Although, overall enzyme performance was not affected by a change in the CO_2 flow rate, a higher triglyceride (TG) and free fatty acid (FFA) fraction and a lower monoglyceride (MG) and diglyceride (DG) fraction were observed at a lower CO_2 flow rate.

The effect of enzyme load, CO_2 flow rate and oil content on the product composition and total production of products was investigated in the on-line extraction-reaction of oil from canola flakes at 24 MPa and 35°C. A higher extent of hydrolysis was observed with an increase in the enzyme load, a decrease in CO_2 flow rate or a decrease in canola load. Scanning electron microscopy (SEM) did not demonstrate any apparent structural changes on the immobilized enzyme.

The diffusion coefficient (D_{12}) is a fundamental parameter in the mass transfer of lipids during supercritical extraction and reactions. D_{12} of several lipid classes was determined in $SCCO_2$ applying the Taylor-Aris peak broadening technique. Presence of a secondary solvent as well as an increase in temperature or a decrease in pressure led to an increase in D_{12} of oleic acid. D_{12} of FFA was reduced by an increase in the

number of double bonds or a change in the position of double bound from ω-3 to ω-6. D_{12} of lipid classes decreased in the following order:

methyl ester > ethyl ester > FFA > TG.

An on-line extraction-reaction process using $SCCO_2$ as developed in this study shows great potential for new process design in the production of high-value products from agricultural commodities, which will be used as ingredients in food and various other industries.

ACKNOWLEDGEMENTS

I would like to express my special thanks to Dr. F. Temelli, my supervisor, for providing the opportunity to learn about the different aspects of supercritical fluids and her on time and invaluable guidance throughout the entire program. I wish her greatest success in all aspects of her life especially in her academic achievements.

I would also like to thank Dr. A. E. Mather, Dr. B. Ooraikul, Dr. M. Palcic, Dr. J. Arul, Dr. P. Sporns and Dr. T. Vasanthan for agreeing to be in the supervisory and/or examining committee, whose input provided more opportunities to learn and contributed to the improvement of this work accordingly. Special thanks are extended to Dr. L. Ozimek for accepting to chair the exam session.

The assistance provided by George D. Braybrook in obtaining the Scanning Electron Microscope images is gratefully appreciated.

I would like to acknowledge the Ministry of Culture and Higher Education of Iran and Natural Sciences and Engineering Research Council of Canada for providing financial support and Novo Nordisk Canada and Canamera Foods for providing enzyme and canola samples, respectively.

Mother's Day

(September 30, 1999, Iranian Calendar)

I gratefully dedicate this work to

my grandmother and

to the spirit of my late mother

who belong to the hard-working and unselfish

Ghashghaei people in Southern Iran

from whom I learnt

how to receive, how to resist and how to respond

to the daily challenges of life.

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NOMENCLATURE

Abbreviations

a.m.u.	Atomic mass unit
AOAC	The Association of Official Analytical Chemists
AOCS	American Oil Chemist's Society
BAUN	Batch Acidolysis Units Novo
FFA	Free fatty acid(s)
FID	Flame Ionization Detector
FTIR	Fourier Transform Infra Red
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
I.D.	Inside diameter
IS	Internal standard
DG	Diglyceride(s)
LD/MS	Laser desorption mass spectrometry
MG	Monoglyceride(s)
O.D.	Outside diameter
RESS	Rapid Expansion of Supercritical Solutions
RSD	Relative standard deviation
$SCCO_2$	Supercritical carbon dioxide
SCF	Supercritical Fluid
SEM	Scanning electron microscopy
SFE	Supercritical fluid extraction
SFR	Supercritical fluid reaction
TG	Triglyceride(s)

Nomenclature for equations

D_{12}	Binary diffusion coefficient
D_{coil}	Coil diameter
De	Dean number
D_{tube}	Column diameter
D_x, D_y, D_{12}	Binary diffusion coefficient
E	Enhancement factor
k	Boltzman's constant
K_i	Inhibition constant of ethanol
$K_{m(et)}$	Affinity constant for ethanol
$K_{m(ol)}$	Affinity constant for oleic acid
K_{ma}	Affinity constant for myristic acid
K_2	Second order rate constant
L	Column length
N_A	Avogadro's number
P	Pressure
P_C	Critical pressure
P_R	Reduced pressure
P_{vp2}	Sublimation pressure for pure solid

pA	Pico Ampere
R	Molar gas constant
r_0	Inside radius of the column
Sc	Schmidt number
T	Temperature
T_C	Critical temperature
T_R	Reduced temperature
t_r	Retention time
U, $\dot{U}_0$, u_0	velocity
$\overline{U}$	Average velocity
$\overline{V}$	Partial molar volume
V	initial velocity
V^s_2	Molar volume of the solid
V_b	Molar volume at the normal boiling point
V_m	Maximum apparent velocity
V_{max}	Maximum initial velocity
$\Delta V^{\pm}$	Activation Volume
$w_{1/2}(t)$	Peak width at half height
y_2	Solubility, mole fraction of a solute in supercritical fluid
ρ	Density
ρ_C	Density at the critical point
ρ_R	Reduced density
ρ_{12}	Approach distance necessary for the reaction, m
Φ	Steric or statistical factor
η	Viscosity
ϕ_2	Fugacity coefficient
ϕ^s_2	The fugacity coefficient at temperature and pressure of the sublimation

1. LITERATURE REVIEW[1]

1.1. INTRODUCTION

Supercritical fluid (SCF) technology is rapidly growing and replacing some of the conventional methods of extraction, reaction, fractionation and analysis. Pressure and temperature dependence of their solvent power has made SCFs very attractive solvents to sequentially extract raw materials from natural sources, conduct reactions of interest, fractionate the products and analyze the fractions in a single unit only by changing the pressure and temperature of the system. Such flexibility is not associated with conventional techniques.

There is a growing demand for flavorings, spices, emulsifiers, preservatives and other basic ingredients of food industry. With the large supply of fats and oils in nature and the possibility of converting them to high value products such as fatty acid esters used as flavorings and mono- and diglycerides (MG and DG, respectively) used as emulsifiers in the food industry, it is quite appropriate to look into some synthetic methods of producing these ingredients.

Other industries such as those of detergents, surfactants, cosmetics and medicine and health related materials highly demand compounds such as specific free fatty acids (FFA), glycerol, MG and DG. New processing techniques to produce such materials need to be evaluated. Organic solvents are used extensively for the processing of the products mentioned above. However, concerns over the use of organic solvents in industrial processing, especially in the food industry, as well as

[1] A version of this chapter is to be submitted to the Journal of Supercritical Fluids for consideration for publication.

environmental issues related to their application for analytical purposes are growing (Lusas and Gregory, 1996; Snyder et al., 1996). Therefore, SCFs are being investigated as alternate media for processing purposes and utilized on an industrial scale.

1.1.1. Current fats and oils technology

Latest developments in technology are always a direct response to the concerns brought about in terms of industry consolidation, customer awareness, environmental issues and agricultural and biotechnological advances etc. (Carlson and Scott, 1991). However, most principles governing fats and oils processing have not changed for years (Carlson and Scott, 1991). Mechanically pressing the oilseeds is the easiest approach to obtain fatty oils (Stahl et al., 1988). To achieve extra recovery from the meal, pressing step is followed by an extraction procedure with a solvent (Stahl et al., 1988). Optimization pretreatment of the starting material can enhance the recovery and the quality of the crude oil. The pressing step usually reduces the oil content to ~20% (w/w). The pressed cake can contain >5% of original oil. It is only after extraction with a lipophilic solvent that the oil content is reduced to <1% (w/w). Hexane is the solvent used in the commercial extraction of oils. During the extraction, the solvent is usually enriched with oil at ~25-35% (w/w) level, which is separated from the solvent by distillation and evaporation processes. The crude oil obtained by extraction is usually cloudy and is not acceptable for consumption. Then, a refining procedure removes the gums, acid, color and the off-odor from the desolventized oil (Stahl et al., 1988). Such a procedure is too long and costly. In addition, with recent

environmental and safety concerns regarding the use of organic solvents, especially hexane (Lusas and Gregory, 1996), a new solvent to replace hexane is being sought. Although diluted hexane and other solvents such as heptane and alcohols are being considered to replace hexane, supercritical fluid extraction (SFE) of oil using a solvent such as $SCCO_2$ is a good alternative to the conventional methods of oil extraction and is gradually becoming more appealing.

1.1.2. Enzyme-catalyzed reactions of lipids

Either chemical catalysts or a biocatalyst may accelerate the process of esterification, which can be used to change the physical and functional properties of edible fats and oils (Yu et al., 1992). Examples of the former include sodium metal, sodium-potassium alloy and sodium alkoxide; sodium methoxide, for instance, has been used in catalyzing the interesterification of saturated long- and short-chain fatty acid triglycerides (TG) in producing new TG for food use (Klemann et al., 1994).

TG or triacylglycerols are the esters of glycerol and have a general structure of $(R_1O)CH_2$-$CH(OR_2)$-$CH_2(OR_3)$ with R_1, R_2 and R_3 representing different acyl groups. Lipase consists of a group of enzymes that catalyze the *hydrolysis* of TG to form FFA, DG, MG and glycerol (Yu et al., 1992). Lipases can also catalyze the following synthesis reactions: *esterification*, combining alcohols or glycerol with fatty acids to form esters; *acidolysis*, the exchange between FFA in fats and oils; and *interesterification*, the exchange of esters between two fats and oils (Yu et al., 1992). The term "*transesterification*" which is often used in the literature can be applied to any of acidolysis or interesterification (Gandhi, 1997).

The full hydrolysis of a TG will produce glycerol and FFA. TG can also undergo partial hydrolysis to produce DG and MG (steps 1-3 below).

Step 1: $TG + H_2O \rightarrow DG + FFA$ (1.1)

Step 2: $DG + H_2O \rightarrow MG + FFA$ (1.2)

Step 3: $MG + H_2O \rightarrow Glycerol + FFA$ (1.3)

Overall reaction:

$TG + 3H_2O \rightarrow Glycerol + 3FFA$ (1.4)

Water is a reactant in hydrolytic reactions as well as a parameter in the catalytic activity of the enzyme (Nakamura, 1994). The extent of hydrolysis; i.e. how much of MG, DG or glycerol is produced, depends on many factors including the water content of the enzyme bed, enzyme morphology, kind of enzyme and pressure and temperature of the reaction. *Glycerolysis* is another reaction of TG, which results in the formation of MG and DG as follows:

$TG + Glycerol \rightarrow MG + DG$ (1.5)

1.2. SUPERCRITICAL FLUIDS

SCFs have received increasing attention as a medium for extraction, reaction and fractionation. This is due to their high diffusivity, low viscosity and marked temperature and pressure dependence of their solvent power. SCFs are solvents at pressures and temperatures above their critical points (Fig. 1.1).

In principle, SCFs are better than conventional solvents to penetrate into the material to be extracted and solubilize the solute (Stahl et al., 1988). SCFs with low critical temperatures such as CO_2 and ethane can be used as solvents in the extraction of heat labile compounds at low temperatures (Stahl et al., 1988). In most cases, SCFs

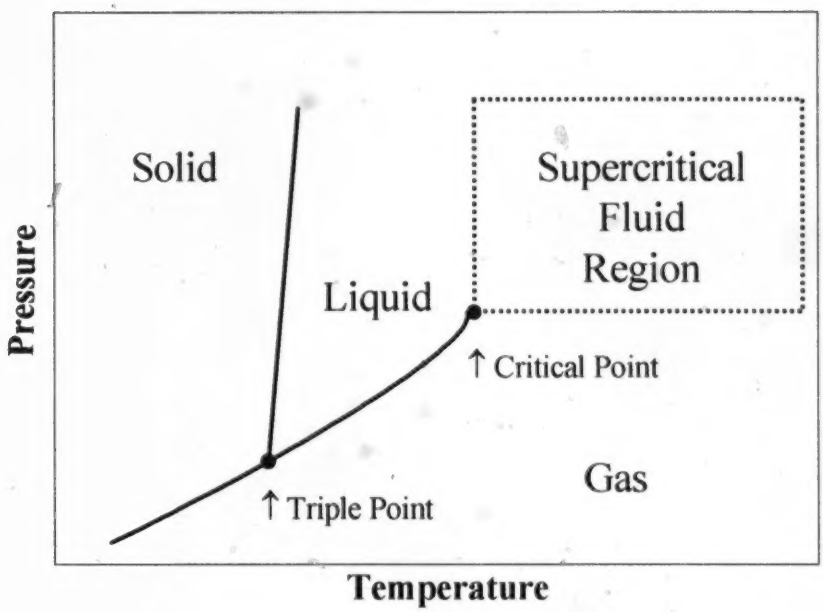

Figure 1.1: A typical phase diagram for most pure substances.

exhibit a high selectivity. Vapor pressure, polarity and molecular weight are parameters that influence the solubility of a compound in SCFs. Non-polar SCFs such as $SCCO_2$ can extract lipophilic compounds, materials with low polarity, particularly those with lower molecular weights and higher volatility (Stahl et al., 1988).

In the vicinity of the critical point, small changes in pressure and temperature cause large changes in the density of a SCF. Thus, the physical properties of a SCF can be manipulated easily and the separation of reaction products (Kamat et al., 1992; 1995a) and the recovery of the unreacted materials can be facilitated in a reactor system.

Having a moderate critical temperature and pressure (31°C, 7.3 MPa) along with its inertness, low price, availability, sterility and safety, $SCCO_2$ has been the solvent of choice for food applications (Mishra et al., 1988; Stahl et al., 1988). $SCCO_2$ provides a non-oxidizing medium for reactions and as a result the production of undesired products is limited. CO_2 can separate as a gas upon pressure reduction without any solvent residue in the final product (Marty et al., 1992b).

1.2.1. Solubility in SCFs

For a binary system, the solubility of a solid solute in a SCF can be obtained from the equation $y_2=(P_{vp2}/P)E$, where P_{vp2} is the sublimation pressure of the solid at the system temperature, P is the total pressure and E is the enhancement factor (Paulaitis et al., 1983). The enhancement factor is the ratio of the solubility in the SCF to that in an ideal gas and can be calculated from the equation $E=(\phi^s_2/\phi_2)\exp[V^s_2(P-P_{vp2})/RT]$, where ϕ_2 is the fugacity coefficient and characterizes the nonideal behavior of the solid in the SCF, ϕ^s_2 is the fugacity coefficient at T and P_{vp2}, V^s_2 is the molar

volume of the solid and T is the system temperature (Paulaitis et al., 1983). Solubility enhancement compared to that in an ideal gas is achieved when $E>1$. The SCF density and molecular interactions between the solute and the SCF are important factors affecting the enhancement factor. Typical enhancement factors in SCFs are of the order of 10^4 (Dinçer et al., 1996). For separation purposes, the partitioning of the solutes between the SCF and the raw material to be extracted is the most important factor. Solubility of the solutes in the SCF has the next priority (Dinçer et al., 1996).

Pressure and temperature influence the density of the SCF, which is an important factor affecting solute solubility. Figure 1.2 demonstrates the changes in the density of CO_2 with pressure at different temperatures. Around the critical pressure (P_c), a drastic change in the density of the solvent occurs with a small change in pressure. Since the solubility of solutes in SCF increases with an increase in solvent density, a dramatic change in the solubility is expected by an increase in pressure from a level below the P_c (i.e. subcritical pressure) to a level above P_c (i.e. supercritical pressure). It is also shown in Figure 1.2 that solvent density decreases with an increase in temperature in the supercritical region.

Although the application of CO_2 alone does not provide good solubility for hydrophilic substrates or products (Knez and Habulin, 1992), adding a secondary solvent into CO_2 can overcome such problems (Stahl et al., 1988). The addition of a secondary solvent can modify the solvent properties so that a selective extraction is obtained. Such solvents are usually referred to as "entrainers" and the terms "co-solvent" and "modifier" are also used. For example, with the addition of acetone or ethanol into $SCCO_2$, the polarity of solvent mixture increases and as a result polar components can be extracted.

Figure 1.2. Variation in the reduced density (ρ_R) of CO_2 as a function of reduced pressure (P_R) and of reduced temperature (T_R) in the vicinity of the critical point (CP). P_c, T_c and ρ_c are the critical pressure, critical temperature and density at the critical point, respectively (McHugh and Krukonis, 1994).

1.2.2. Diffusivity of solutes in SCFs

The binary diffusion coefficients of solutes in SCFs play a major role in their extraction from the solid matrix as well as their dissolution from liquid mixtures for fractionation purposes. Also, in the enzyme-catalyzed reactions of substrates in SCFs the binary diffusion coefficient is an important parameter to determine how well a compound can migrate to the active site of enzyme as well as how well a product leaves the enzyme site to reach the bulk SCF.

When the rate of a SCF process (extraction, reaction or fractionation) is dependent on the diffusion of solutes through the solvent environment, a control on diffusion coefficient can improve the process rate by manipulating the parameters that can enhance the diffusion step. When two or more solutes are present in a mixture, a control on the diffusion coefficient of each solute can optimize their separation.

The Taylor-Aris peak-broadening technique is a method to determine the diffusion coefficient of solutes in a solvent. When a pulse of a solute is introduced into a solvent stream flowing through a straight tube under laminar conditions (Fig. 1.3), it broadens into a peak as a result of the combined effects of convection along the tube axis and molecular diffusion in the radial direction (Liong et al., 1991). The velocity profile of the solvent in laminar flow is shown by arrows parallel to the tube axis where the velocity is highest at the tube center. Solutes diffusing in the radial direction and moving down the tube with solvent flow result in a broad peak which can be detected with a detector if the tube is long enough. Taylor-Aris peak-broadening technique is a correlation between the peak width and the solute diffusion coefficient, which is presented in detail in chapter 5.

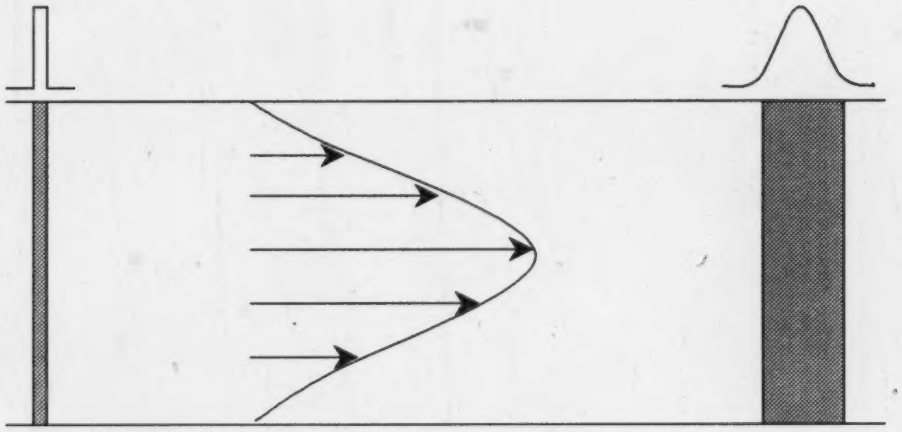

Figure 1.3. Schematic of a peak broadening under a laminar flow of the solvent stream.

At a given temperature, diffusivity of a dilute solute in SCFs is typically an order of magnitude higher than that in a liquid (Clifford, 1994). Increasing temperature enhances the diffusivity as a result of a decrease in the solvent viscosity. The relationship between the diffusion coefficient and viscosity is given by the Stokes-Einstein equation (eqn. 1.6), which has demonstrated a good agreement with experimental results (King and Catchpole, 1993)

$$D_{12} = kT / 3\pi\eta(V_b / N_A)^{1/3} \qquad (1.6)$$

where k is the Boltzman's constant, T the absolute temperature, η the viscosity of the medium, V_b the molar volume at the normal boiling point and N_A the Avogadro's number. Temperature also affects the density of the SCF. An increase in temperature will lead to a lower density of the SCF and as a result in a higher diffusivity of the solutes. Pressure is another factor affecting the diffusivity of solutes in the SCFs. Its influence is described by an effect on both density and viscosity of the solvents. Increasing pressure will result in a decrease in the diffusion coefficient of the solute. This is due to an increase in the collision frequency of the solute molecules, which results in a reduced mean path of the molecules.

The rate determining step for some fast reactions is the rate at which the substrates are diffusing in the solvent (Clifford, 1994), because that is the step which controls the access of substrates to the point where they react. In such a case, the following equation can relate the second order rate constant (k_2) with the diffusivity of the solutes assuming only two substrates are involved in the reaction (Clifford, 1994):

$$k_2 = 4\pi N_A \rho_{12} \phi (D_X + D_Y) \qquad (1.7)$$

where D_X and D_Y are the binary diffusion coefficients of the substrates in the fluid

studied, ρ_{12} is the approach distance necessary for the reaction and ϕ is a steric or statistical parameter less than unity.

1.3. SUPERCRITICAL FLUID EXTRACTION

Fats and oils are conventionally extracted using organic solvents such as hexane. However, with the latest changes in the regulations regarding the use of organic solvents, they are being banned for applications in food processing and also their use in analytical procedures is restricted (Lusas and Gregory, 1996; Snyder et al., 1996). As a result, supercritical fluid extraction (SFE) is receiving increasing attention. Industrial plants performing extraction and fractionation of flavors and spices from natural matrices in SCFs are available.

Extraction with SCFs has been used as an alternative to the conventional methods of solvent extraction, vacuum distillation and thin film evaporation (Stahl et al., 1988). Technically, an SFE operation involves the flow of a fluid through a solid matrix held in an extraction chamber at a pressure and temperature above the critical point of the solvent. When the SCF is in contact with the material to be extracted, depending on the solute's partitioning between the two phases, the solutes can be dissolved into the SCF and carried away from the solid matrix. The solute(s) can then be separated from the SCF by releasing the pressure.

Preconditioning of the samples is one of the most important steps determining the yield and quality of the extract (Stahl et al., 1988). One or more of the following steps may be involved in the preconditioning: pulverization, peeling, cell cracking, flaking and cold pressing. Depending on the solute(s) of interest, the choice of solvent and entrainer is another important step. For example, using a mixture of propane and

CO_2 in the extraction of oil from colza (a rapeseed variety) resulted in a higher solubility than that in CO_2 alone (Stahl et al., 1988). Other process parameters such as extraction time, solvent flow rate, presence of an entrainer such as water or ethanol are also important in determining the extraction rate and composition of the extracts.

Pressure and temperature are the main operational parameters in controlling an SFE process. Generally, the solubility of fats and oils is increased by an increase in both pressure and temperature (Stahl et al., 1988). Therefore, if a maximum extractability is required, higher pressures and temperatures are applied, in which case due to the presence of the pigments, the extracts are usually colored (Dinçer et al., 1996). A colorless product can be obtained if milder operating conditions are used. One application of extractions at low pressure and temperature is to remove the undesirable odors from such products as vegetable oils (Dinçer et al., 1996). To perform a fractional extraction of the components, a stepwise pressure increase can be applied.

1.3.1. Extraction kinetics

Figure 1.4 shows a typical curve for the extraction of lipids from solid matrices. Kinetically, there are three steps involved in the process of extraction throughout the entire run. In the first step, there is an equilibrium between the lipids in the SCF and the lipids in the solid matrix (Reverchon, 1997). Presence of equilibrium is justified by the linearity observed in the first part of the graph indicating that there is no control on the extraction rate from internal diffusion of the lipids in the solid phase. Increasing the solvent flow rate in this stage of extraction will result in a higher extraction rate since the equilibrium will provide the necessary oil to saturate the SCF. In the transition phase, oil freely available on the surface of solid particles is being

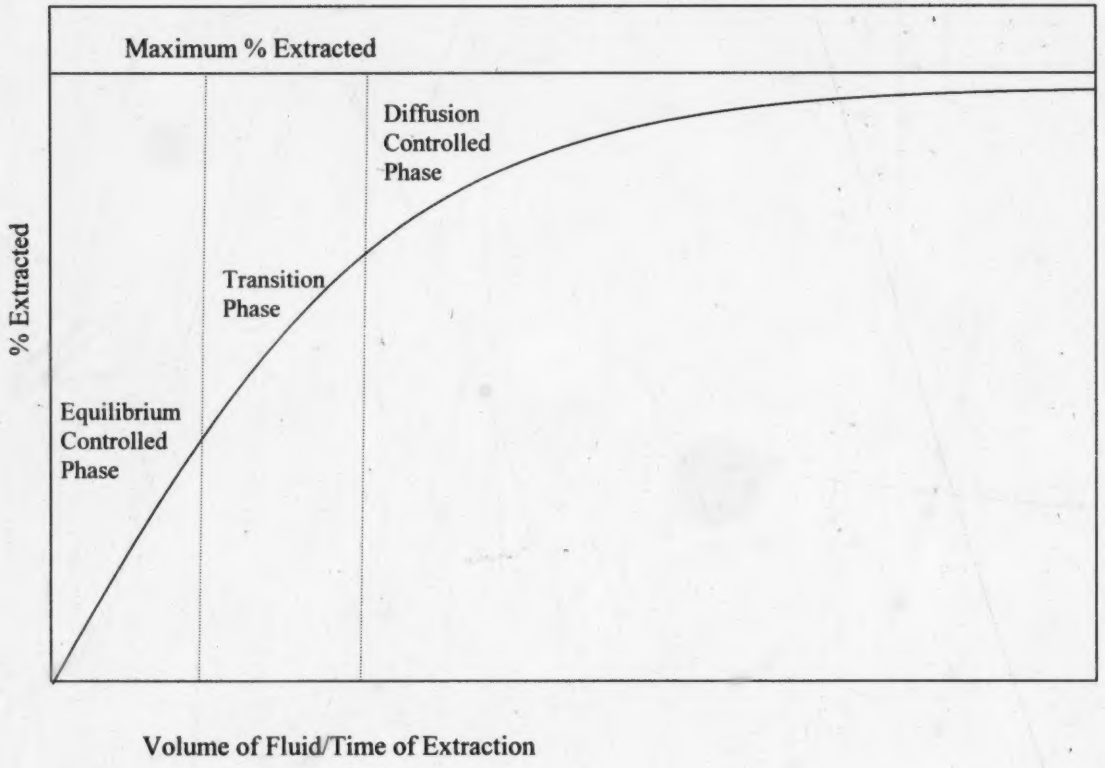

Figure 1.4. Typical extraction curve.

depleted and saturation cannot be maintained, thus internal diffusion starts to control the extraction rate. On the last portion of the graph, a plateau is observed which indicates that the extraction rate is exclusively controlled by the diffusion rate of the lipid through the solid matrix.

The slope of the linear portion of the curve in Figure 1.4 can be used to obtain the solubility of lipids at a given pressure and temperature condition. External mass transfer in an extraction process is restricted by the solubility of solutes in the SCF and as a result only a higher solvent flow rate can increase the extraction rate of the solutes. This indicates that oil is available on the surface of the solid particles where it can be easily removed. However, when the oil is located in internal pores and cells, internal mass transfer resistance controls the extraction rate. In this case, the effect of solvent flow rate on the kinetics of the extraction process is negligible (Reverchon, 1997).

1.3.2. Supercritical fluid extraction of lipids

SFE has been applied to the extraction of many lipid products including cholesterol, and fats and oils. Also, it has been used in the extraction of other components of oilseeds, such as phospholipids, FFA, waxes, and substrates imparting odor, taste and color. Extraction of edible oils from different sources such as soybean, canola seed, cottonseed and other oilseeds has been performed using $SCCO_2$ (Dinçer et al., 1996). To deacidify edible oils, $SCCO_2$ alone was able to separate TG and FFA since their vapor pressures and solubilities are different (Stahl et al., 1988). The following are some lipid related applications of SFE (Stahl et al., 1988).

1- Defatting of snacks such as potato crisps, containing 40-50% frying fat and converting to a product more digestible and lower in calories with a longer shelf-life.

2- Extraction of TG, FFA, waxes and cholesterol from pasteurized egg yolk powder leaving a protein product with ~20% phosphatidylcholine.

3- Extraction of anhydrous animal fats from butcher-shop residues by static contact with a SCF for 10-60 min.

Economically, a countercurrent extraction process is more advantageous since the saturation solubility of SCF can be maintained. To ease the loading and unloading of solid materials containing lipids, a cage screw extruder can introduce the raw material into the high pressure system and take the residue out of the extractor without the need to release the pressure of the system (Stahl et al., 1988).

1.4. ENZYMATIC REACTIONS IN SUPERCRITICAL FLUIDS

Conducting reactions in supercritical media is an area that experienced rapid growth over the last decade since higher conversion rates could be achieved compared to conventional media. A review of the literature, though not comprehensive, is presented in Table 1.1 for enzymatic reactions in $SCCO_2$. Similarly, Tables 1.2 and 1.3 present examples of non-enzymatic reactions in $SCCO_2$ and reactions carried out in a supercritical fluid other than CO_2, respectively. The discussion in this section will focus on enzymatic reactions in $SCCO_2$ medium.

Selectivity, specificity, milder reaction conditions and tolerance to water and fatty acids are the advantages that make enzymatic reactions more attractive than chemical methods (Erickson et al., 1990). Enzymes can maintain their activities in a

Table 1.1. Examples of enzymatic reactions in $SCCO_2$.

Reaction	Enzyme	Condition	Remarks	References
Regioselective oxidization of phenols into their corresponding benzoquinones	Polyphenol oxidase	36°C and 34 MPa	70-80% conversion in 40 min	Hammond et al. (1985)
Formation of *p*-nitrophenol from disodium *p*-nitrophenyl phosphate	Alkaline phosphatase	35°C, 101 MPa and 0.1 vol. % water content	~53% conversion in 4 h	Randolph et al. (1985)
Synthesis of the precursors of aspartame	Thermolysin	40°C and 20.3 MPa	33% conversion in 2h reaction followed by 1 h extraction	Kamihira et al. (1987)
Oxidation of cholesterol with molecular oxygen	Cholesterol oxidase from 1- *Streptomyces* sp., 2- *Pseudomonas sp.* and 3- *Gleocysticum chrysocreas*	35°C and 98.7 MPa	Conversions 1- 11% in 2 h, 12-14% in 24 h 2- 100% in 24 h 3- 70% in 13 s	Randolph et al. (1988)
Transesterification of ethyl acetate and isoamyl alcohol	Liposyme TM	60°C and 10 MPa	100 g isoamyl acetate or 200 g nonyl acetate at optimum water content of 45-60 g per kg of immobilized enzyme	van Eijs et al. (1988)
Transesterification between N-acetyl-L-phenylalanine chloroethyl ester and ethanol	Subtilisin	45°C and 15.0 MPa in the presence of 0.5-5% ethanol	10-54% conversion in 15 min 90% conversion at 2.5% ethanol in 30 min	Pasta et al. (1989)
Interesterification between tricaprylin and methyl oleate with shifting the equilibrium by removing methyl oleate	Lipase from *Mucor miehie*	40°C and 10 MPa	87.3% conversion in 14 h	Adschiri et al. (1992)
Hydrolysis of cellulosic material Avicel after a $SCCO_2$ pretreatment	Cellulase (reaction not in supercritical condition)	35-80°C and 6.9-26.6 MPa as pretreatment condition	A pretreatment at temperatures above the T_c of CO_2 and 20.7 MPa pressure provides a glucose yield of ~70% (w/w) in 23 hours.	Zheng et al. (1995)

Table 1.1. Examples of enzymatic reactions in $SCCO_2$ (Cont'd).

Reaction	Enzyme	Condition	Remarks	References
Esterification between oleic acid and oleyl alcohol	Lypozyme IM and Palatase 1000L (immobilized and free, respectively, lipases from *Mucor miehie*)	40°C and 15.0 MPa	- In a batch system, conversion is 40-50%. - In a continuous system, at constant flow rate of CO_2 (0.8 L/min), conversion declines, from ~33% at 0.14 L/min to ~12% at 0.35 L/min, as flow rate of substrate increases.	Knez et al. (1995)
Esterification of myristic acid with ethanol	Immobilized lipase from *Mucor miehie*	50°C and 12.5 MPa	- The internal mass transfer limitation in *n*-hexane as a solvent was compared with that in $SCCO_2$. - In hexane, the reaction rate was controlled by diffusion rate of myristic acid while in $SCCO_2$, an intermediate rate between that controlled by reaction kinetics and the one obtained from diffusional control was achieved.	Bernard and Barth (1995)
Enzymatic alcoholysis of cod liver oil	Immobilized lipase from *Candida antarctica*	40°C and 9-24 MPa	- 25% conversion at 40°C and 9 MPa	Gunnlaugsdottir and Sivik (1995)
Peptide synthesis from N-acetyl-L-tyrosine ethyl ester and several amino acid amides; glycinamide (Gly-NH_2), alaninamide (Ala-NH_2), valinamide (Val-NH_2) and leucinamide (Leu-NH_2) in acetonitrile (MeCN)/$SCCO_2$ system	Bovine pancreatic α-chymotrypsin	35°C and 20 MPa	-Water content and $SCCO_2$/MeCN ratio had significant effect on the yield of the reaction. After 5 h of reaction, the yield was - 64% in MeCN system. - 91% in 20%(v/v) $SCCO_2$ in MeCN/$SCCO_2$ system. - reduced at higher MeCN/$SCCO_2$ ratios and as hydrophobicity of the side chain of amino acid amide increased; that is, GLy- > Ala- > Val- > Leu-NH_2	Noritomi et al. (1995)
Synthesis of oleic acid esters from oleic acid and different primary alcohols	Immobilized lipase from *Rhizomucor miehei* [Lyposyme IM]	20-100°C and 50 MPa	Depending on the alcohol used, a conversion of 80-94% was obtained at 50°C, optimum temperature.	Habulin et al. (1996)

Table 1.1. Examples of enzymatic reactions in $SCCO_2$ (Cont'd).

Reaction	Enzyme	Condition	Remarks	References
Avicel hydrolysis into glucose	Cellulase	35-50°C and 3.45-13.8 MPa	- At 46°C and 6.7 MPa (subcriritical condition), the optimum yield of ~70% (w/w) is obtained. - Increasing pressure at 46°C increases the yield (~60% at 3.45 MPa, ~70% at 6.7 MPa and ~75%(w/w) at 13.8 MPa after 24 h).	Zheng et al. (1996)
Transesterification of lipids by methanol	Lipase from *Candida antarctica*	50°C and 17.24 MPa	- A conversion of 99.5% or better can be obtained.	Snyder et al. (1996)
Esterification of different secondary alcohols with acetic anhydride	Immobilized lipase from *Pseudomonas sp.*	40°C and 20 MPa	- For most secondary alcohols tested, the rate of the reaction in the $SCCO_2$ is higher than that in all organic solvents used. - Similar results were obtained for the enantiomeric excess values.	Catoni et al. (1996)
Transesterification of ethyl acetate and isoamyl alcohol as a model reaction in designing a SCF extraction process for separating mixtures incurred in enzyme-catalyzed reactions	Lipase	N/A	For an efficient and cost-effective separation of reaction components, - Separate the fractions of components that have significantly different solubilities in the SCF first. - Then, depending upon the vapor phase solubilities of components, choose appropriate temperature and pressure and the number of theoretical stages, for a desired purity of the component of interest. - For a complete separation, avoid the alyiotropic region in the system.	Chrisochoou and Schaber (1996)
Transesterification of soybean oil with glycerol, 1,2-propanediol and methanol	Lipase from *Candida antarctica*	40-70°C and 20.7-34.5 MPa	- Optimum pressure and temperature were found to be 27.6 MPa and 70°C, respectively. - Conversion of 1,2-propanediol was increased by increasing the flow of soybean oil (>90% yield of monoglycerides and monoester can be obtained). - Reaction more likely takes place in a multiphasic mixture.	Jackson and King (1997)

Table 1.1. Examples of enzymatic reactions in $SCCO_2$ (Cont'd).

Reaction	Enzyme	Condition	Remarks	References
Transesterification between triolein and stearic acid	Lipase	50°C and 5-10 MPa	- In subcritical region, reaction rate was maximum at 5.9 MPa - In supercritical region reaction rate increased with an increase in pressure	Nakaya et al. (1998)
Transesterification of *n*-butyl acetate by 1-hexanol	Novozym 435	35°C and 10 MPa	- Reaction rate in propane and ethane was higher than that in CO_2	Almeida et al. (1998)
Hydrolysis of tripalmitin and fats	*Candida antarctica* lipase	60°C and 27.4 MPa	- Moisture content of the enzyme was important factor in the hydrolysis reaction	Hampson and Foglia (1999)

Table 1.2. Examples of non-enzymatic reactions in $SCCO_2$.

Reaction	Remarks	Reference
Oxidation of cumene (isopropylbenzene).	· Neat oxidation; that is, when no supercritical solvent is used and reaction is done in liquid cumene, has given better yields than oxidations in the mixture critical region.	Suppes et al. (1989)
Effect of the extraction of furfural on the selectivity and yield of furfural production from xylose	· Furfural was recovered to a great extent under supercritical conditions due to the suppression of side-reactions such as polymerization and decomposition.	Sako et al. (1992)
Esterification of oleic acid by methanol	· *p*-Toluenesulfonic acid, homogeneously, and the cation-exchange resins K2411 and K1481, heterogeneously, have been used as catalysts.	Vieville et al. (1993)
Telomerization of tetrafluoroethylene employing perfluorobutyl iodide as telogen	· $SCCO_2$ showed to be an exceptionally interesting solvent to study free radical telomerization of tetrafluoroethylene, particularly in the production of perfluoroalkyl iodides with a narrower and more controlled range of molecular weights.	Romack et al. (1995)
The heterogeneous polymerization of bicyclo[2.2.1]hept-2-ene and norbornene using $Ru(H_2O)_6(tos)_2$ as initiator	· The NMR spectra from the products showed that CO_2 does not incorporate into the polymer, but the microstructure of the polymer changes dramatically compared to that obtained in other solvents. · Quantitative addition of methanol can control the microstructure of the carbon dioxide/methanol systems.	Mistele et al. (1995)
Glycerolysis of vegetable oils to monoglycerides	· For soybean oil, at 250°C, 20.7 MPa, 4% water content and with a glycerol/oil ratio of 25, a maximum of 49.2% monoglyceride and 14% FFA were obtained after 4 h of reaction. · Among five different oils (soybean, peanut, cottonseed, corn and canola) soybean and cottonseed oils provided the highest and the lowest conversion to monoglyceride, respectively, although the conversion values were very close to each other.	Temelli et al. (1996)

Table 1.3. Examples of reactions carried out in a supercritical fluid other than CO_2.

Reaction	Medium	Remarks	References
Hydrolysis of wood cellulose into glucose using solutions of 1% to 33% water in sulfur dioxide	Near- and supercritical sulfur dioxide	· A yield of up to 2% glucose and 21% xylose was obtained at near critical condition (170°C and 8.3 MPa).	Vick Roy and Converse (1985)
Inverse microemulsion polymerization of acrylamide	Near- and supercritical alkane continuous phases	· The structure of the precursor microemulsion was found to be strongly dependent on the density of the continuous phase.	Beckman and Smith (1990)
Water oxidation of dissolved phenol	Water	· The influence of slow and fast preheating of the feed wastewater and the concentration of substrate on the supercritical and subcritical conditions at 315-426°C and 24.2 MPa in a tubular reactor was investigated.	Li and Egiebor (1994)
Sequential conversion of citric acid to itaconic acid and then to methacrylic acid	Near- and supercritical water	· The yield of methacrylic acid, which reached a maximum of about 70% (mole basis) of the itaconic acid feed, was dependent on the temperature, pH and buffer strength of the medium.	Carlsson et al. (1994)
Wet air oxidation	Water	· The mechanism, kinetics and structure-oxidizability correlation for wet air oxidation of carboxylic acids, phenols, cyanides and nitriles were reviewed.	Mishra et al. (1995)
Oxidation of acetic acid	Water	· For the oxidation of acetic acid in supercritical water, the global oxidation rate law is first order in acetic acid, 0.6 order in oxygen and second order in water. Pre-exponential factor and activation energy for this reaction are $10^{19.8}$ $M^{-2.6}$ S^{-1} and 73.6 kcal/mol, respectively.	Savage and Smith (1995)
Synthesis of boron nitride from boron oxide	Ammonia	· B_2O_3 (solid) was converted to hexagonal boron nitride at a temperature below the melting point of B_2O_3 using supercritical ammonia and solid CaO. · No product was produced at conditions below the critical point of ammonia, that is 132.4°C and 11.2 MPa.	Yokoi et al. (1995)

wide range of temperature, pressure, pH and salt concentrations and in different environments such as aqueous solutions, organic solvents and supercritical media (Vermuë and Tramper, 1995). By studying the enzymatic transesterification of methylmethacrylates as their model reaction in supercritical ethane, ethylene, fluoroform, sulfurhexafluoride and near-critical propane, Kamat et al. (1992) showed that the reaction rate achieved in all the SCFs studied was significantly greater than that in any conventional solvent tested. The finding that biocatalysts, especially enzymes, can maintain their activity at high pressures has encouraged the use of enzymes to catalyze reactions under supercritical conditions. By introducing a controlled amount of water to the delicate structure of enzymes, SCFs can be used as media where the enzyme kinetics can be correlated with solvent properties, such as dielectric constant and hydrophobicity, without changing the solvent but by controlling pressure and/or temperature only (Kamat et al., 1992; 1995a). In the enzyme-catalyzed synthesis of polymers in SCFs, the molecular weight of the products was controlled by a change in the system pressure only (Russell et al., 1994; Chaudhary et al., 1995). Furthermore, SCFs have an extra advantage that the products can be separated from each other under supercritical conditions. For example, the separation of mono-, di- and triolein in $SCCO_2$ was possible by manipulating pressure and temperature only (Stahl et al., 1988).

1.4.1. Factors influencing enzymatic reactions in SCFs

1.4.1.1. Water content

Water is one of the reactants in hydrolytic reactions. In addition, as an entrainer of the SCF, it can modify the polarity of the environment and thus the solubility of solutes. Also, the enzyme activity depends on the amount of water present

in the SCF medium. In the complete absence of water, enzymes are inactive (Kamat et al., 1995a). However, if the water content is more than its optimum value, roughly 10% (w/w) for both *n*-hexane and $SCCO_2$, it has a negative effect. Marty et al. (1992a) observed a decline in the residual activity of Lipozyme after conducting reactions in the presence of different water levels for one day. The enzyme appeared to be irreversibly denatured in the presence of excess water. Marty et al. (1992a) suspected this effect to be due to some undesirable reactions such as hydrolysis of enzyme proteins. When a large amount of water is added, water is adsorbed on the enzymatic support, and the enzyme gets fully hydrated by many layers of water. This phenomenon results in a decrease in the enzyme activity since the layers of water act as a barrier between the enzyme surface and the reaction medium (Dumont et al. 1992). Chi et al. (1988) reported an increase in the initial rate of the lipase-catalyzed interesterification of triolein with stearic acid and hydrolysis of triolein with an increase in water content in both *n*-hexane and $SCCO_2$. They considered water to act as a modifier of the solvent having a one hundred fold higher solubility in $SCCO_2$ than in *n*-hexane. Nakamura (1994) studied the interesterification of triolein and stearic acid in a continuous reactor, at 20-30 MPa and 40-70°C. He reported that the formation of mono- and di-substituted TG increased with water concentration followed by a slight decrease; however, a larger amount of water was required to get the optimum productivity at shorter residence times. Similar to Chi et al. (1988), Marty et al. (1990) reported that the initial rate collapsed rapidly in the esterification of oleic acid and ethanol once the water content reached 12% (w/w of enzyme support) for *n*-hexane and 110% for $SCCO_2$.

Trace levels of water were found to be necessary for catalysis by maintaining the active conformation of the enzyme molecule through hydrogen bonding (Marty et al., 1992*a*; Caralp et al., 1993; Nakamura, 1994; Perrut, 1994). Enzyme shape can change depending on the extent of hydrogen bonding produced by water molecules inside and outside the enzyme (Clifford and Bartle, 1996). Also, depending on the polarity of the microaqueous layer around the enzyme and the polarity of the reaction components, enzyme can react differently. Since the microaqueous layer around the enzyme is responsible for enzyme activity, any change in the physical properties of the microqueous layer such as pH, size, shape and solubilizing activity can influence the reaction.

Contrary to Marty et al. (1990; 1992a), Yu et al. (1992) showed that adding 1 mL water to 1 g immobilized *C. cylindracea* lipase at 40°C and 13.6 MPa resulted in a maximum conversion of 30% for the reaction of ethanol and oleic acid to produce ethyl oleate. Yu et al. (1992) concluded that the enzyme support adsorbed some water because of its hydrophilic nature. The hydrolysis of ethyl oleate to ethanol and oleic acid was possible when there was excess water on the immobilized lipase. As well, Yu et al. (1992) achieved 10% hydrolysis of methyl oleate in 30 min at 40°C and 13.6 MPa at similar enzyme and water concentrations.

Vermuë et al. (1992) studied the transesterification of nonanol and ethyl acetate to form nonyl acetate and ethanol in near-critical CO_2 using *Mucor miehei* lipase. They investigated the effects of pressure, polarity and water content of the medium on the reaction in a continuous stirred-tank reactor. The pressure and polarity of the near-critical CO_2 hardly influenced the transesterification rate of lipase, but

changing the water content of the system from 0.05 to 0.2% (v/v) caused a decrease in product formation. The transesterification rate in near-critical CO_2 was lower than that in hexane.

Marty et al. (1992a) determined the adsorption isotherm of water between the enzymatic support, macroporous anionic resin beads, and solvent so that the activity in $SCCO_2$ could be better compared with that in *n*-hexane. They observed a negative temperature effect on the adsorption of water on the solid support, since more water will be in the supercritical phase than on the solid phase with increasing temperature; i.e., the partition coefficient gets larger, as it is usual for gases. A similar result was obtained with increasing pressure, which is contrary to what one would expect from the adsorption isotherms, since the effect of the vapor pressure of water between the two phases was overcome by the solvating effect of supercritical medium on water. Usually, one layer or less of water around each enzyme molecule is essential to maintain the native structure of the enzyme and thus its activity (Kamat et al., 1995a). Hydrophilic organic solvents absorb this very layer of water and often denature the enzyme. Table 1.4, which was derived from the results of Kamat et al. (1992), indicates that the activity of enzyme decreases with an increase in water partitioning from the enzyme into the hydrophobic solvents. For example, they measured an increase in the concentration of water in hexane and butyl ether of 0 and 1.5μL/mL, respectively, after the reaction was completed. Kamat et al. (1992) considered the water in butyl ether to be essentially partitioned from the enzyme into the solvent, since the only source of water in the reaction system was that of the enzyme. When anhydrous (0.01% water) acetonitrile and dioxane were used as solvents there was no

Table 1.4. Effect of solvent in the alcoholysis of methylmethacrylate by 2-ethylhexanol (100 mM each) at 30°C catalyzed by lipase (20 mg/mL) from *Candida cylindracea*.[a]

Solvent	Dielectric Constant	Initial rate (mM/h-mg)
Dodecane	2.01	0.234
Hexane	1.89	0.193
Butyl ether	3.12	0.170
Butyl acetate	5.01	0.044
Methylene chloride	9.08	0.008

[a]From Kamat et al. (1992).

detectable reaction rate, whereas rates of ~20 μmol/min per mg of enzyme were achieved upon water addition (25 μL/mL solvent).

Dumont et al. (1992) investigated the effect of water on the rate of esterification of myristic acid and ethanol by adding different amounts of water to the substrates and showed that the initial rate could be increased by a factor of 2-3. The maximum initial rates exhibited for $SCCO_2$ and *n*-hexane were at 25-55 mM and 1.1 mM water, respectively, above which initial rate decreased substantially. Once again, the higher partition constant of water in $SCCO_2$ was demonstrated.

Kamat et al. (1995a) considered using salt hydrates to control the amount of water delivered to the reaction system. A hydrated salt can give off water to the environment based on an equilibrium and maintain the water activity at a certain level. As water is consumed by the reaction system, new molecules of water are released from the salt to compensate for the lack of water activity in the environment. Different hydrated salts at different temperatures can be used to meet the water requirement of various reaction systems. $Na_4P_2O_7.10H_2O$ was used at 35.17 MPa in supercritical fluoroform by Kamat et al. (1995a) to study the effect of salt hydrate on the

transesterification rate of N-acetyl-L-phenylalanine ethyl ester by methanol using subtilisin as catalyst. Saturation of the supercritical medium occurred at a minimal salt concentration of 1 g/40 mL to reach the maximum enzyme activity (Kamat et al., 1995a). Such a test with salt hydrates has not been carried out for $SCCO_2$.

Another negative effect of extra water is its contribution to decreasing enzyme thermostability. Water is believed to increase the mobility of protein molecules and enhance its unfolding rate (Kamat et al., 1995a). Also, the presence of water initiates disulfide interchange, glutamine and asparagine deamination and the hydrolysis of peptide bonds (Kamat et al. 1995a). Therefore, not surprisingly, enzyme thermostability in nonaqueous media is higher than that in aqueous solutions. In fact, while lipase has a lifetime of 12 h in tributyrin (with 0.015% v/v water) at 100°C, its lifetime is only a few seconds in water at the same temperature (Kamat et al. 1995a). For optimum enzyme activity and stability, water content has to be adjusted according to the operating temperature.

The hydrolysis of triolein and its partial glycerides was studied by Glowacz et al. (1996) using porcine pancreas lipase in $SCCO_2$. It was shown that the enzyme activity and the enantiomeric ratios of the products were dependent on the water content of the enzyme, substrate and the reaction time. For example, when the enzyme water content was 5 or 15%, a decrease in the hydrolytic activity in the order: triolein > *rac*-1,2-diolein > 1,3-diolein > *rac*-1-monoolein could be observed, while this order was not observed when the enzyme water content was 1.5%.

1.4.1.2. Type of enzyme

Different kinds of enzymes can target different reactions. Michor et al. (1996) investigated the resolution of racemic citronellol and menthol using enzyme-catalyzed

transesterification in $SCCO_2$. The activities of different lipases, AY30 from *Candida rugosa*, PS from *Pseudomonas sp.*, Novozyme 435 from *Candida antarctica* and immobilized Lipozyme IM60 from *Mucor miehei*, were compared with the activity of esterase EP10 from *Pseudomonas marginata*. The reactions were carried out at 50°C and 10.0 MPa, except for lipase PS whose reaction temperature was 35°C. For the transesterification of (±)menthol using isopropyl acetate as an acyl donor, esterase gave higher enantioselectivity and initial rate than all lipases studied. When the enzymes lipase AY30 and esterase EP10 were used (±)citronellol exhibited higher initial rates than (±)menthol, 110 and 120 $\mu mol\text{-}h^{-1}g^{-1}$, respectively. Neither lipase AY30 nor esterase EP10 showed any enantiomeric preferences (Michor et al., 1996). Liu et al. (1997) reported that for the synthesis of cocoa butter equivalent immobilized lipase from *Mucor miehei* was the most efficient and specific among the five different lipases studied.

1.4.1.3. Selectivity of the enzyme

The selectivity of enzymatic reactions is an advantage that is very difficult to obtain by chemical methods. The reactivity of a few racemic alcohols such as (±)-1-phenylethanol, (±)-2-octanol in $SCCO_2$ using immobilized crude lipase (*Pseudomonas sp.* from Amano) has shown that a high enantiomeric excess of >80% can be approached by enantioselective acetylation of racemic alcohols (Cernia et al., 1994). Rantakylä et al. (1996) reported the hydrolysis of racemic 3-(4-methoxyphenyl) glycidic acid methyl ester in $SCCO_2$ using immobilized lipase from *Mucor miehei* as catalyst. The presence of the enzyme was essential for the reaction to take place. As soon as water was injected into the reactor, both (2S,3R)- and (2R,3S)- forms started

to react. However, the hydrolysis rate for the former was faster than that of the latter. During the first 40 min of the reaction, the initial reaction rates were 250 and 44 mg/g enzyme per hour for (2S,3R) and (2R,3S) forms, respectively. The lipase-catalyzed enantioselective esterification of glycidol in $SCCO_2$ was conducted by Martins et al. (1994). Esterification of glycidol with butyric acid catalyzed by porcine pancreatic lipase immobilized on Sephadex G-25 or Bio-gel P6 led to an enantiomeric purity of 83±2% of (S)-glycidyl butyrate at 25-30% conversion and 20-25% hydration of the enzyme preparations. The selectivity achieved was similar to the highest value ever obtained in organic solvents.

The kinetic resolution of 3-hydroxyhexanoic acid, 3-hydroxyoctanoic acid and 3-hydroxy decanoic acid methyl esters in *n*-hexane and $SCCO_2$ catalyzed by lipase from *Pseudomonas cepacia* was investigated by Capewell et al. (1996). They transesterified the 3-hydroxy esters either at the free hydroxyl group using different esters or at the ester group using ethanol. The highest enantiomeric excesses and enantioselectivities were found for the reactions of 3-hydroxyoctanoic acid methyl ester, particularly for its reaction with styryl acetate, where enantiomeric excess was 99% (R) after 47 h indicating a preference of the enzyme on specific compounds.

An interesting approach in the area of selective enzymatic esterification in $SCCO_2$ was developed by Castillo et al. (1994) and applied to the esterification of glycerol with oleic acid. Phenylboronic acid was used as a complexing agent to protect positions 1 and 2 on the glyceride chain leaving only one position available for esterification and glycerol was immobilized on silica gel. The immobilized lipase from *Mucor miehei* Lipozyme TM was used as catalyst. Even though the formation of only

monoolein was expected, diolein was produced as well. A significant conversion in the esterification reaction occurred with a higher concentration of monoolein.

1.4.1.4. Pressure and temperature

Both pressure and temperature affect the density and transport properties of the SCF, which in turn affect the solubility and transport of reactants and products to and from the enzyme by the SCF, respectively. Pressure and temperature can also affect enzyme activity (Ishikawa et al., 1996) and reaction thermodynamics. The esterification of a primary terpene alcohol such as citronellol was performed with oleic acid in the presence of lipase in $SCCO_2$ (Ikushima et al., 1995b). An optical purity of almost 100% was obtained from a racemic compound, (±)citronellol, by only manipulating the pressure and temperature in the vicinity of the critical point of carbon dioxide.

To determine the effect of pressure on enzyme activity, Balaban et al. (1991) treated orange juice samples containing their natural pectinesterase with $SCCO_2$ at 13.7 and 31 MPa and 40-60°C. Pectinesterase activity was reduced to a level at which the orange juice maintained its cloudy appearance longer than when it was untreated. Although temperature was a factor in reducing pectinesterase activity, temperature effect was not significant if high pressure were not applied (Balaban et al., 1991). The treatment with high pressure CO_2 involved reducing pH due to the formation of carbonic acid from the dissolution of carbon dioxide in water. The orange juice exhibited pH's as low as 2.96 at 31 MPa and 35°C. Balaban et al. (1991) suggested that the low pH might be the main parameter in reducing pectinesterase activity and not the high pressure since the enzyme restored its activity after the pressure was

released. However, there are some enzymes that are active at pH's as low as 2. For example, lipase from *Candida cylindracea* is active over the pH range of 2-8.5 (Yu et al., 1992). In addition, Owusu-Yaw (1988) showed that only when the pH of orange juice was dropped to ~2.0 by the addition of HCl or when the orange juice was treated with acidic cationic resins, could substantial reduction in pectinesterase activity take place.

Kamat et al. (1992; 1995b) have shown that CO_2 covalently bonds to enzymes and temporarily inactivates them; therefore, reducing pH by itself may not be responsible for any reversible inactivation. Also, Randolph et al. (1991) reported that conformational changes due to the changes in pressure were minimal, based on the results of high-pressure electron paramagnetic resonance spectroscopy of cholesterol oxidase from *Gloecocysticum chrysocreas* in $SCCO_2$ and $SCCO_2$/entrainer mixtures. A similar result was obtained for lipase from *Rhizopus arrhizus* (Miller et al., 1991). Shishikura et al. (1994) experienced that the immobilized *Mucor miehei* lipase could be stable for more than 180 h in $SCCO_2$ at 60°C and 9.8 MPa.

Pressures beyond a certain level, however, might have some negative effects on the enzyme itself. For example, Randolph et al. (1991) mentioned enzyme denaturation at ultra-high pressures, e.g. >400 MPa, which was probably due to the direct effect of high pressure on the conformation of enzyme molecules.

Pressure can affect the reaction rate by changing the concentrations of reactants and products in solution since partitioning of reaction components between the two phases depends on pressure (Erickson et al., 1990). Pressure may as well have an effect by influencing the mass transfer; i.e., due to changes in density, viscosity, diffusivity etc., and by altering the rate constant of the reaction (Randolph et al.,

1991). Immobilized enzyme is not considered to be responsible for these variations (Erickson et al., 1990).

1.4.1.5. Effect of $SCCO_2$ on enzyme activity

The nature of the SCF and whether it has any physical or chemical influence on the solutes is an important factor since the SCF interact with the reaction components and enzyme and thus may affect the reaction rate. CO_2 at high pressures may have some effects in addition to the effects related to the high pressure itself. When seven free amino acids were exposed to $SCCO_2$, there was no change in their structure except for glutamine, which partially reacted with CO_2 (Weder, 1984). *L*-Arginine displayed no changes over the pH range of 3-7 when it was exposed to $SCCO_2$ at 30 MPa and 80°C for 6 h (Weder et al., 1992). However, at pH 9, a ≥10% conversion to arginine bicarbonate was observed. In agreement with Kamat et al. (1992; 1995b), there might have been some reversible changes during the exposure time which could not have been detected by the methods used by Weder et al. (1992), .i.e., thin-layer chromatography, ninhydrin reaction, etc. Using Laser Desorption Mass Spectrometry, LD/MS, Kamat et al. (1995b) showed that the enzyme was modified covalently and the covalent bond was demolished upon pressure release. There might be a significant pH or CO_2 effect which is overcome by the effects of change in other parameters such as pressure and temperature.

Lozano et al. (1996) reported a positive effect due to pressure on the activity of α-chymotrypsin. Their studies on gas, liquid and supercritical media showed that the half-life of enzyme increased proportionally with an increase in the density of CO_2 in any medium. They suggested that the increase in the density of a fluid could be used

as a means of protecting enzymes from deactivation. Lozano et al. (1996) related this important phenomenon to the increase in several parameters such as dielectric constant, polarity/polarizability and/or Hildebrand solubility parameter of the solvent. These parameters have a direct effect on the hydrophilic property of the solvent resulting in enhanced enzyme stability by promoting solvent-protein interactions (Vermuë et al., 1992; Lozano et al., 1996). Ikushima et al. (1995*a;* 1996) suggested that the SCF itself can have some additional effects. In a given SCF, solubilities of compounds depend on pressure, temperature and density of SCF as well as the structural parameters of the solute such as chain length, degree of unsaturation, molecular weight and the presence of various functional groups (Yu et al., 1994).

By monitoring the enzyme conformation using Fourier Transform Infrared, FTIR, spectroscopy in $SCCO_2$, Ikushima et al. (1995a) showed that over a limited range of pressure, 7.7-8.5 MPa, dramatic changes in *C. cylindracea* lipase occurred resulting in a higher reaction rate. The pressure range of esterification reactions where lipase from *C. cylindracea* catalyzed the production of optically active compounds overlapped with the pressure range in which sudden conformational changes took place (Ikushima et al., 1995a). When conducting lipase-catalyzed esterification of *n*-valeric acid and citronellol in $SCCO_2$, Ikushima et al. (1996) observed a sharp increase in the reaction rate at pressures close to the critical region of CO_2. They speculated that the electron acceptability of $SCCO_2$ becomes appropriate at a given pressure to conduct a particular esterification reaction like that of *n*-valeric acid and citronellol. However, Ishikawa et al. (1995) reported a slow decrease in the activities of glucoamylase and acid protease with a change in $SCCO_2$ density up to a certain point called the intersection point (0.82 and 0.60 g/cm^3, respectively), after which

enzyme activity showed a sudden drop. Similar trends were observed with an increase in temperature or pressure. Although the results of Ikushima et al. (1995*a*; 1996) and Ishikawa et al. (1995) do not appear to be in agreement, these results belong to different enzymes at different pressure ranges (*Candida cylindreacea* lipase at 7-8 MPa for the former and glucoamylase at >15 MPa and acid protease at >8.5 MPa for the latter). Nakamura et al. (1991) applied a gravimetric method to measure the adsorption isotherms for CO_2 on several proteins such as casein, gelatin, gluten and ovalbumin. They observed a sharp peak in the adsorption isotherms of all the proteins studied just above the critical pressure of CO_2 (8.3-8.8 MPa), which is very close to the pressure range reported by Ikushima et al. (1995a; 1996).

1.4.1.6. Reactor design

Whether a batch or a continuous system is used and how each component of the reactor is designed will influence the reaction flow. A batch system is usually used for kinetic studies. The advantage of a continuous reactor over a batch system other than having a consistent flow is continuous withdrawal of the products which may prohibit propagation of the thermodynamic equilibrium towards higher conversion. Accumulation of some products such as FFA can inhibit enzyme activity (Lencki et al., 1998). Briand et al. (1995) demonstrated that the presence of oleic acid in an aqueous medium at 4.0 μM fully inhibited the enzymatic activity of *Candida parapsilosis* in the hydrolysis of rapeseed oil and oleic acid ethyl ester. However, for the methanolysis of rapeseed oil only 45% activity loss was observed. In order to maintain the driving force of the reactions, continuous removal of inhibiting products such as FFA is necessary, which cannot be done with a batch system.

1.4.1.7. Enzyme support

Enzyme support has been shown to play an important role in the catalytic activity of immobilized enzymes. Kind of enzyme support used, its adsorption isotherms and stability throughout the reaction are important parameters affecting the overall activity of the enzyme. Enzymes in their non-immobilized form; i.e., free enzymes, have different sizes, polarizability, chemical structure and protein content and therefore exhibit different properties in terms of their specificity and selectivity in catalyzing different reactions. For application purposes, free enzymes are dissolved in a solvent such as water or an organic solvent. However, in the immobilized form, enzymes are bound on a solid matrix such as glass beads (Miller et al., 1991) and natural or synthetic materials including bioskins (Legaz et al., 1998), silk fibroin membrane (Liu et al., 1995) and polyaniline films (Verghese et al., 1998). Enzymes can be attached to the solid support by different ways including ionic attractions and covalent bonds. Depending on immobilization method, enzymes may loose part of their activity or become more stable after immobilization.

1.4.1.8. Other parameters

There are numerous other factors that affect enzymatic reactions carried out in SCF media. The following are the most important ones:

1) The solubility of the reactants and the products in SCF. More soluble reactants can reach the enzyme sites more easily and as a result a more favorable reaction is expected. Similarly, if a product can leave the enzyme site fast, the reaction can proceed further.

2) The dielectric constant of the SCF. This is a major factor in dissolving substrates and carrying them through the reaction system, which is affected by many other parameters such as pressure, temperature, presence of an entrainer, etc.

3) Type of the reaction, i.e. hydrolysis, esterification, transesterification etc. A given set of conditions may be more favorable for one type of reaction than others. For example, the presence of water at a certain level may promote a hydrolysis reaction and not esterification.

4) Morphology of the enzyme. Enzymes have specific morphology which may be influenced by different parameters such as pressure and temperature. As well, an enzyme may be immobilized on different supports which can affect various properties of the enzyme.

5) Enzyme load. The amount of enzyme used and how it is placed in the reactor influence the reaction results. For example, if packing inhibition is not a concern, the material throughput can be increased by using larger quantities of the enzyme.

6) Type and specificity of the substrate, its molecular weight, chain length and number and position of double bonds, if any. The properties of the substrate play a major role in all reactions including enzymatic reactions in SCFs.

7) The thermodynamic equilibrium of reaction and how the components of the reaction medium, particularly water in hydrolytic reactions, will partition between the phases. It is important to have the substrates and solvent in a single phase where the reaction has to take place.

8) The presence of side reactions. Usually, side reactions take place parallel to the main reaction in each process. The extent of side reactions at the conditions of the study will determine the conversion rate for the target reaction.

10) Interaction between the factors discussed above. Some or all of the factors may in one way or another be correlated with each other.

1.4.2. Enzyme activity

Enzymes are complex protein molecules, which are chains of amino acids, joined by peptide bonds (Krawczyk, 1997). The tertiary structure of the enzyme molecules that is held together by hydrogen bonding, ionic and hydrophobic interactions as well as disulfide bridges determine how the peptide chains are arranged in three dimensional space. Enzymes with S-S bridges are more stable compared to those without such covalent bonding.

Enzymes exhibit different activity levels depending on the substrate and environmental conditions. Yu et al. (1992) reported 30% and 75% conversions for the synthesis of ethyl oleate and octyl oleate, respectively, at 8.2 MPa and 40°C using immobilized lipase from *Candida cylindracea* as a catalyst in $SCCO_2$. As well, a conversion rate of 100% was reported in the synthesis of ethyl butyrate (Gillies et al., 1987) using 5 g immobilized lipase from *C. cylindracea* in 100 mL *n*-heptane containing 0.25 mol/L butyric acid and 0.40 mmol/L ethanol after 22 h. The reaction times in the first two cases where $SCCO_2$ was used were shorter, ~1 h, and the initial velocities were higher, compared to the last one using a conventional solvent, 22 h. When conversions obtained in $SCCO_2$ (Yu et al., 1992) were compared to those obtained in *n*-hexane (Lazar et al., 1986), Yu et al. (1992) argued that regardless of

the solvent used for given reactants, similar conversions should be achieved. They also concluded that a conversion of 95% in the synthesis of ethyl butyrate should be expected in $SCCO_2$ using a similar lipase from *C. cylindracea*. Reaction medium is another factor in the catalytic activity of the enzymes. Although lipases and proteases catalyze esterification reactions in aqueous solutions, alcoholysis is a preferred reaction in a non-aqueous environment (Kamat et al., 1992).

The enzyme powder recovered from the alcoholysis reaction of methylmethacrylate and 2-ethylhexanol in $SCCO_2$ at 11 MPa and 45°C was active in the same alcoholysis reaction after slow depressurization (Kamat et al., 1992). Similar results were obtained in *n*-hexane, in which CO_2 had been introduced. Kamat et al. (1992) concluded that inhibition imposed by carbon dioxide on the enzymes was reversible in both cases of hexane and $SCCO_2$.

There are two controversial arguments regarding the way enzyme inactivation takes place. One is that rapid release of CO_2 dissolved in the bound water, due to a decrease in pressure, causes a structural change in the enzyme leading to its inactivation (Nakamura, 1990). The second argument considers that the inactivation can also take place during pressurization. Zagrobelny and Bright (1992) investigated the conformational changes in the trypsin using fluorescence spectroscopy. They reported that trypsin stability was decreased during pressurization.

1.4.3. Kinetics of enzymatic reactions

The enzymatic esterification of oleic acid and ethanol catalyzed by Lipozyme, immobilized lipase from *Mucor miehei*, in $SCCO_2$ was compared to that in *n*-hexane by Marty et al. (1992a). The enzyme exhibited good stability in both media depending

on the moisture content of the solid enzyme. Due to the complexity of the problem, Marty et al. (1992a) considered obtaining the optimum enzyme conformation to be the most important role of water and did not take into account the water influence during initial rate formulation. Different concentrations of ethanol were used to determine the role of ethanol in the reaction mechanism. A ping-pong bi-bi mechanism prohibited by ethanol was suggested to be involved in the reaction. A reciprocal coordinate representation of initial velocities against acid concentration, where parallel lines are obtained at low concentrations of ethanol, could easily describe this mechanism. The slopes of these parallel lines increased with increasing ethanol concentration, and their intercepts approached a limiting value of $1/V_{max}$. The reaction mechanism seemed to be similar in both $SCCO_2$ and *n*-hexane. Figure 1.5 depicts the reaction scheme suggested by Marty and co-workers (1992a). A substrate-enzyme complex is produced by linkage of oleic acid to the enzyme which in a later step delivers water. Ethanol is then linked to the remaining species producing another complex, which finally gives rise to ethyloleate and is released from the enzyme. The other parallel pathway, which is not favorable, is the linkage of ethanol directly to the enzyme producing an inactive complex. The resulting equation from the model of Segel is written as follows to describe the above mechanism (Marty et al., 1992a):

$$\frac{V}{V_{max}} = \frac{[Ol][Et]}{K_{m(ol)}[Et]/K_i + K_{m(et)}[Ol] + [Ol][Et]} \qquad (1.8)$$

where $[Ol]$ and $[Et]$ are the initial concentrations of oleic acid and ethanol, and $K_{m(ol)}$ and $K_{m(et)}$ are their respective affinity constants, K_i is the inhibition constant of ethanol, and V and V_{max} are the initial and maximum initial velocities, respectively (Marty et

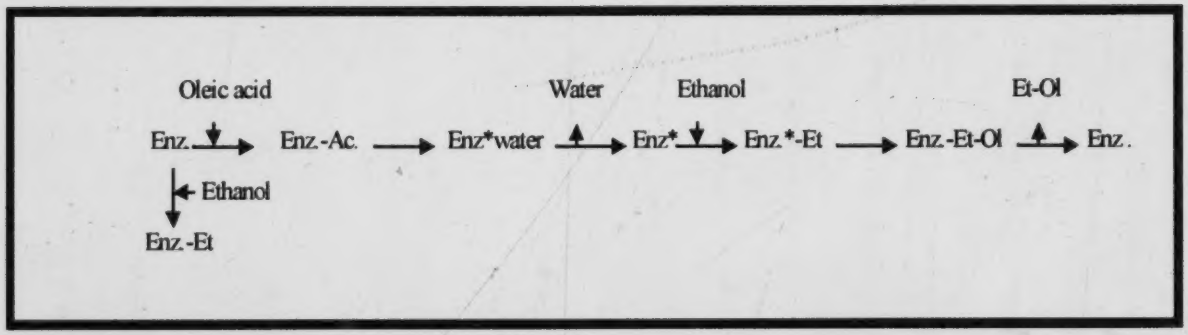

Figure 1.5. Schematic representation of the ping-pong bi-bi mechanism with inhibition by ethanol suggested by Marty et al. (1992a). Enz. = Enzyme, Ac. = (Oleic) Acid, Et = Ethyl, Ol = Oleate and Et-Ol = Ethyloleate.

al., 1992b). A plot of the reciprocal of initial rate versus the acid concentration will give the limiting value of the intercept, $1/V_{max}$, and the plot of slopes versus [*Et*] will give the inhibition constant for ethanol, $K_{i(et)}$ and affinity constant of oleic acid for Lipozyme, $K_{m(ol)}$. A direct parametric identification on the model equation (Gauss-Newton algorithm) and derivation of the apparent kinetic parameters resulted in 5.3% average relative error and 15.7% maximum error between experimental and calculated data. Table 1.5a gives a comparison of these parameters for $SCCO_2$ and *n*-hexane. Maximum apparent velocity (V_m) seems to be higher in *n*-hexane than that in $SCCO_2$, possibly because of the pH change in the aqueous phase surrounding the enzyme, since CO_2 can diffuse into the trapped microaqueous phase in the enzymatic support and acidify the medium. Thus, the enzyme conformation may have been affected.

Dumont et al. (1992) studied the behaviour of Lipozyme in $SCCO_2$ and *n*-hexane catalysing the reaction of myristic acid and ethanol at different substrate concentrations. The resultant kinetic constants are listed in Table 1.5b. Dumont et al. (1992) considered a ping-pong bi-bi mechanism for the reaction of myristic acid with ethanol, in either *n*-hexane or $SCCO_2$. Maximum velocity in $SCCO_2$ was 1.5 times higher than that in *n*-hexane, while the affinity constant in $SCCO_2$ was lower for myristic acid ($K_{ma,\ SCCO2} < K_{ma,n\text{-hexane}}$). They concluded that inhibition in $SCCO_2$ is smaller than that in *n*-hexane since ethanol acted as a co-solvent and enhanced the myristic acid solubility in $SCCO_2$ by increasing the polarity of the medium. Both ethanol and water are more soluble in $SCCO_2$ than in *n*-hexane. Dumont et al. (1992) also concluded that the entrainer effect of ethanol caused the drying of the enzymatic support. This is in contrast to the findings of Marty et al. (1992a) who showed that the negative effect of ethanol still existed even after the addition of 15% water. If the dryness of the enzyme were the problem, it should have been resolved by addition of extra

TABLE 1.5. Kinetic constants for the enzymatic esterification of oleic and myristic acids with ethanol obtained in *n*-hexane and $SCCO_2$.

Parameters	Solvent	
	$SCCO_2$	*n*-Hexane
a. Oleic acid + ethanol (From Marty et al., 1992a)		
V_m (mmol/min-g)	14	23
$K_{m(ol)}$ (mM)	170	450
$K_{m(et)}$ (mM)	1600	600
K_i (mM)	65	60
b. Myristic acid + ethanol (From Dumont et al., 1992)		
V_{max} (mol/min-mg)	0.833	0.532
K_i (mM)	120	43
K_{ma} (mM)	9.2	12.8

V_m = Maximum apparent velocity, $K_{m(ol)}$ and $K_{m(et)}$ = Affinity constants for oleic acid and ethanol, respectively, and K_i = Inhibition constant of ethanol.
V_{max} = Maximum initial velocity, K_i = Inhibition constant of ethanol and K_{ma} = Affinity constant for myristic acid.

water to the batch reactor, but this was not the case.

1.4.4. Influence of phase behavior on the reaction rate in SCFs

It is very important to know what the phase behavior of a reaction system is so that a single supercritical phase is obtained; i.e., a homogeneous phase rather than two or more heterogeneous phases. Modifying the pressure and temperature of a SCF system can lead to a homogeneous phase and therefore a productive reaction. The solubility of the reactants and products in the SCF and the possibility of a phase separation are the factors that can determine whether a reaction system can proceed efficiently. Reaction rates can be higher in the mixture critical region (McHugh and Krukonis, 1994). Phase equilibria for enzyme-catalyzed reactions in $SCCO_2$ were studied by Chrisochoou et al. (1995) using the lipase-catalyzed transesterification of

ethyl acetate and isoamyl alcohol to produce ethanol and isoamyl acetate as a model reaction. They applied the Soave-Redlich-Kwong equation of state with the mixing rules of Huron and Vidal to predict vapour liquid equilibrium behavior of the system and found that the Soave-Redlich-Kwong model, which was first written for a two-component system, was capable of predicting high pressure phase equilibria of multicomponent systems over a wide range of conditions. Phase equilibria obtained in this way can be used in designing SCF extraction, reaction and fractionation processes to optimize operating conditions.

1.4.5. Effect of activation volume on reaction rate

Applied pressure as well as partial molar volume can have a great effect on the reaction rate in supercritical media (Combes et al., 1992; McHugh and Krukonis, 1994). Transition state analysis can be used to describe the rate increase occurring at high pressures. The variation of the reaction rate constant, k, with pressure for a bimolecular reaction represented by the equilibrium equation "A + B = $M^{\pm}$ = Products" is as follows:

$$\left(\frac{\partial \ln k}{\partial P}\right)_T = \frac{\Delta V^{\pm}}{RT} \qquad (1.9)$$

where "$\Delta V^{\pm} = \overline{V}_M - \overline{V}_A - \overline{V}_B$" is the activation volume; i.e., the difference in the partial molar volumes of the activated complex, $M^{\pm}$, and the starting materials, A and B. This equation considers the reaction rate in terms of mole fractions. When reaction rates are expressed in concentration units, an extra term in the equation accounts for the compressibility of the reaction mixture (Wu et al., 1991; Boock et al., 1992; Caralp et al., 1993; Clifford, 1994; Srinivas and Mukhopadhyay, 1994; Kamat

et al., 1995a). The activation volume in liquid solvents is ±50 mL/mol and that in SCFs is at least two orders of magnitude higher (Kamat et al., 1995a).

If the activation volume in a reaction is positive; that is, if the activated complex has a higher partial molar volume than the reactants, then increasing pressure will not favor the reaction. However, with a negative activation volume, where the reactants are more voluminous than the activated complex, the reaction rate will increase with pressure. Care must be taken since partial molar volumes are very sensitive to any change in pressure, temperature and mole fraction in the vicinity of the critical point of the reaction mixture or the critical conditions of the solvent, when a dilute solute is used. This fact was displayed in the results of Srinivas and Mukhopadhyay (1994) in the oxidation of cyclohexane to cyclohexanol and cyclohexanone in $SCCO_2$. For example, an increase in temperature favored the production of the latter more than the former. Increasing the pressure and temperature reduced the induction period for the production of both products. Activation volumes calculated for the oxidation of cyclohexane at 17.0 MPa changed from 36 to -775 cm^3/mol with an increase in temperature from 137 to 160°C indicating that increasing pressure at 137°C will not result in any improvement in the reaction rate, since the activation volume is positive. However, the reaction will proceed at a much higher rate at 160°C because of its negative activation volume at this temperature. Srinivas and Mukhopadhyay (1994) showed that the feed composition had a great influence on the reaction rate and considered this parameter as the main possible reason for the activation volume to be so high at 137°C. The activation volume for dilute systems is dictated by i) the differences in the van der Waals attractive and repulsive forces of the

reactants and the activated complex with the solvent, and ii) the isothermal compressibility of the pure solvent (Combes et al., 1992; Srinivas and Mukhopadhyay, 1994).

1.5. RESEARCH NEEDS

Future applications of enzymatic reactions in SCFs include lipid reactions, such as hydrolysis, (trans)esterification and the synthesis of TG; aroma compound synthesis; and enantiomer purification and/or stereoselective synthesis as suggested by Perrut (1994). For food applications, the production of specific fatty acid esters similar to the flavorings used in margarine, imitation dairy products, confections and other processed foods is possible with enzymatic esterification. Enzyme-catalyzed transesterification has been used to modify the physical and functional properties of fats and oils in manufacturing high-value confectionery fats by exchanging fatty acids between fats or oils using lipase. Yu et al. (1992) investigated the applicability of the immobilized *Candida cylindracea* lipase in producing ethyl oleate in $SCCO_2$ and then applied the optimum conditions in this reaction to synthesize the ethyl esters of fatty acids of milk fat. Liu et al. (1997) synthesized cocoa butter equivalent using the enzyme catalyzed interesterification of TG high in POP (P, palmitate; O, oleate) and POO in $SCCO_2$. The hydrogenation of oils to produce margarine and production of nutritionally important TG such as those containing ω-3 fatty acids are among other possibilities.

There is great potential for developing new food processes involving extraction/reaction/fractionation schemes. Ingredient industry, especially fats, oils and flavors, is going to benefit from such on-line operations, where the specifications

needed for ingredients can be met using proper fractionation in such sequential designs. Rapid Expansion of Supercritical Solutions (RESS) is another process (Turk, 1999) that can be combined with one or more of the above-mentioned processes in order to produce solid particles with different specifications required for a given food application. Therefore, the possibilities of using $SCCO_2$ for process development are almost endless and working in this rapidly growing area is very rewarding. All of these processes involve the manipulation of parameters such as the pressure and temperature of the SCF. The following is intended to highlight the areas which need further consideration:

- Despite the presence of many SCFs with low critical points (Table 1.6), not many studies have been conducted to support enzyme-catalyzed reactions in these media. Some immediate concerns are the flammability of several of them and a higher price in cases like neon.

Table 1.6. Critical temperature (T_c) and pressure (P_c) of some gases.[a]

Gas	T_c (°C)	P_c (MPa)
CO_2	31.0	7.36
C_2H_6	32.2	4.88
C_3H_8	96.8	4.26
C_2H_4	9.9	5.11
C_3H_6	91.9	4.60
Ne	-228.76	2.76
N_2O	36.5	7.27
SF_6[b]	45.6	3.77

[a]From Weast (1988).
[b]From Stahl et al. (1988).

- Physical properties of SCFs need to be properly correlated with the activity, specificity and stability of the enzymes. Some supercritical solvents such as sulfur hexafluoride (SF_6) show high potential as a medium for conducting enzymatic reactions (Kamat et al., 1992).

- Different parameters affecting enzyme activity and those affecting the physical properties of the supercritical solvent must be independently studied and every reaction (hydrolysis etc.) for a given enzyme or a group of enzymes be investigated separately. For instance, the pressure effect in an enzymatic transesterification may not be the same as that in a hydrolysis reaction of the same enzyme. In fact, since enzymes catalyze both direct and reverse reactions in a given system, some opposite results should be expected when performing a direct reaction like hydrolysis compared to a reverse reaction like transesterification. Thus, compilation of a database that allows an appropriate comparison of the effect of different parameters on a certain system is needed and can lead to a better understanding of the effects and their interactions.

- More attention is required towards some enzymes that have proven to be stable in $SCCO_2$ but do not seem to be employed in enzymatic reactions to a great extent in such a medium.

- The activity of enzymes has been limited by an increase in temperature and/or pressure. To raise this limit, genetic engineering needs to produce enzymes with higher tolerance for high pressure and temperature.

- Selective esterification of glycerol with oleic acid by immobilization and complexation of glycerol was a successful application (Castillo et al., 1994). It seems

quite appropriate to look into other reactions with such complexing agents as phenylboronic acid since they appear to be very helpful in limiting unnecessary by-products.

- Conducting the on-line processes of supercritical fluid extraction of substrates, supercritical fluid reaction, fractionation and analysis of the products is a possibility that at this point is in its earliest stages of development. Such an approach was applied in small scale for on-line SCF extraction/SCF reaction by Jackson and King (1996) and for on-line SCF extraction/SCF reaction/gas chromatography by Snyder et al. (1996). As well, Chrisochoou and Schaber (1996) reported some procedures to facilitate such an approach. Extraction of food materials followed by their reaction shows great potential in food industry.

- Use of salt hydrate as a source of water in the enzymatic reactions seems to be an appropriate means of controlling the water concentration of SCFs. Food technology lacks the research involving the application of such an approach in $SCCO_2$.

1.6. OBJECTIVES OF THE THESIS

Canola is an oilseed crop originated in Canada as the name denotes (*Canadian + oil => Canola*). In 1978, the name canola was given to any of the cultivars *Brassica napus* and *B. campestris* to distinguish them from the other rapeseed species (Canola Council of Canada, 1982). *B. napus* and *B. campestris* are species which can lead to an oil product containing <%5 erucic acid and not more than 3 mg glucosinolates in 1 g of the moisture-free, oil-free meal. Both erucic acid and glucosinolates are the antinutritional components of canola seed. The quality of canola

oil is good from several points of view including its low content of saturated fats, ~7% (w/w), and high content of linolenic acid (an ω-3 fatty acid), 11% (w/w) (Canola Council of Canada, 1994). Among the crops grown in Canada, canola is the second highest revenue source of the country, after wheat. Because of colder temperature and soil requirements for canola crop, Canada is a good choice to grow this crop, which is evident by its large production level, > 6 million tons/year. Thus, research on canola oil and its value-added processing aspects has major economical advantages for the country.

Conducting reactions in SCFs has gained increasing attention and achievements in this area are growing. As well, the separation of reaction products and the recovery of unreacted materials have become possible in a supercritical reactor system.

Fast growing enzyme technology and the possibility of using enzymes at high pressures such as those in the SCFs have opened new areas of research. This has a potential for replacing organic solvents by environmentally safer SCFs. Although, there are numerous SCFs to be used as solvents to conduct enzymatic reactions (Table 1.6), due to lower price, safety and inertness, $SCCO_2$ has been the most commonly used solvent in research and industry. It has been used for extraction and fractionation as well as in conducting enzymatic reactions.

MG and DG are important ingredients of many food products for emulsification and stabilization purposes. The traditional method of synthesizing these materials is the glycerolysis of oils, which involves the use of alkali metals at high temperatures, >250°C (Temelli et al., 1996). In addition, the product is a mixture of

MG, DG, and FFA and their metal salts. Separation of MG and DG from such a mixture is performed by distillation and the final products very often have a dark color as well as undesirable flavor and odor (Temelli et al., 1996). However, MG and DG can be produced using enzymatic hydrolysis or enzymatic glycerolysis of edible oils at significantly lower temperatures (close to room temperature) in SCF media. Furthermore, there is potential for selective preparation and purification of each product by adjusting reaction conditions such as pressure, temperature, solvent flow rate, enzyme load, etc.

FFA and glycerol, which are other products of hydrolysis reaction, are used in the production of many products such as detergents, cosmetics, surfactants, medical and health related materials. A batch reactor system was used for the hydrolysis of triolein and its partial glycerides using porcine pancreas lipase in $SCCO_2$ (Glowacz et al., 1996). It was shown that the enzyme activity and the enantiomeric ratios of the products were dependent on the water content of the enzyme, substrate and the reaction time. Edible oils are abundantly available in nature in many oilseeds including canola, soybean, corn and sunflower, which are currently extracted using the traditional methods employing *n*-hexane. Such a process is very lengthy and the cost for the solvent is high. As well, the ever-rising concerns about the use of *n*-hexane in terms of air polution and safety issues are pushing the governments and industry to reduce its use and find an alternative. With the latest developments in SFE technology and its growing involvement in many processes including those in the flavors and spice industry, it is quite appropriate to incorporate such a technique to extract fats and oils directly from their sources and convert them to higher value products through an on-line enzymatic reaction. Recently, such on-line enzymatic reaction of extracted

materials was reported (Jackson and King, 1996; Snyder et al., 1996). However, both the extraction and reaction processes were incorporated in a single cell so that it was not possible to apply different conditions for each process. There are no reports in the literature of an on-line extraction-reaction process where extraction and reaction are carried out in two separate cells with individual control on pressure, temperature and moisture content. Developing such an on-line connection between the extractor and the reactor will simplify conventional processing steps and as a result will save time, energy and other resources.

Numerous studies have reported a loss in enzyme activity and/or stability after exposure to certain conditions such as high pressure (Kamat et al., 1992; Yu et al., 1992; Lee et al., 1993) or excessive water content (Marty et al., 1992a). However, our understanding of structural changes that may occur when an enzyme is exposed to high pressure in supercritical media is quite limited. The literature lacks information on the structural changes of immobilized enzymes exposed to supercritical conditions.

When dealing with extractions, reactions and fractionations in SCFs, mass transfer information is essential to explain transport phenomena happening in such systems. The binary diffusion coefficient of compounds in SCFs is an important parameter for optimal process design. Such data for a limited number of lipid components have been reported at low pressures (Catchpole and King, 1994; Dahmen et al., 1990, Funazukuri et al., 1989,1991,1992; Liong et al., 1991,1992). However, extraction of lipids from natural matrices using $SCCO_2$ is usually carried out at pressures >25.0 MPa to achieve higher solubility levels. The literature lacks information on the diffusion coefficients of lipid components at these conditions.

The overall objective of this thesis work was to develop an on-line extraction-

reaction system to extract oil from oilseeds and convert them to high value products using supercritical CO_2. Lipase-catalyzed hydrolysis of canola oil was selected as a model reaction. The specific objectives to achieve such goal were:

1) to investigate the effect of pressure, temperature and CO_2 flow rate on the conversion and product composition in the lipase-catalyzed hydrolysis of canola oil in $SCCO_2$ using a continuous flow reactor (chapter 2);

2) to investigate the effect of CO_2 flow rate, enzyme load and the quantity of canola flakes on the composition of the product mixture in the on-line extraction-reaction of canola oil from canola flakes (chapter 3);

3) to study the effect of high pressure, CO_2 flow rate, enzyme load and oil content on the enzyme structure using a scanning electron microscopy (chapter 4); and

4) to determine the diffusion coefficients of several classes of lipids at pressures up to 36.0 MPa and to investigate the effect of pressure, temperature, presence of a secondary solvent/solute and the number and position of double bonds on the diffusion coefficient of lipids in $SCCO_2$ (chapter 5).

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2. LIPASE CATALYZED HYDROLYSIS OF CANOLA OIL IN SUPERCRITICAL CO_2[1]

2.1. INTRODUCTION

Supercritical fluids (SCFs) have received increasing attention as a medium to conduct enzymatic reactions since high reaction rates can be achieved. Their higher diffusivity and lower viscosity compared to organic solvents make them more attractive as reaction media to transport reactants and products to and from the enzyme. Furthermore, the marked temperature and pressure dependence of their solvent power eases the post-reactional separation of the products.

The conventional method of triglyceride (TG) hydrolysis is the Colgate-Emery steam splitting process. In this method, superheated (>250°C) steam at about 5 MPa pressure (Linfield et al., 1984) is used. Such high temperatures can lead to oxidation, dehydration and interesterification of lipids (Gandhi, 1997).

Some fatty acids, especially those with a higher degree of unsaturation, cannot withstand such high temperatures and as a result will decompose to products such as ketones and hydrocarbons. On the other hand, enzymatic hydrolysis of fats and oils is superior since the reaction is performed under much milder conditions with fewer hazards and lower power consumption (Gandhi, 1997). As well, enzymatic reactions are selective, side reactions are restricted, and thus are best suited for the production of specific compounds where products have better color and odor (Gandhi, 1997).

Mono- and di-glycerides (MG and DG, respectively), free fatty acids (FFA)

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[1] A version of this chapter is to be submitted to the Journal of the American Oil Chemists' Society for consideration for publication.

and glycerol can be produced using enzymatic hydrolysis of edible oils. MG and DG are important ingredients used in numerous food products for emulsification purposes. Free fatty acids (FFA) and glycerol are used in the production of many products such as detergents, cosmetics, surfactants, medical and health related materials. Supercritical CO_2 ($SCCO_2$) has been used for the extraction and fractionation of lipids (Mishra et al., 1988, Hampson et al., 1996). Carbon dioxide with a moderate critical condition, 7.4 MPa and 31°C, is a suitable solvent for fats and oils. For food applications CO_2 is the preferred solvent. Recently, it has been studied as a medium to conduct enzymatic reactions. Many enzyme-catalyzed reactions have proven successful in SCF media. Enzymes were shown to maintain their activity at pressures as high as 400 MPa (Randolph et al., 1991). Running enzymatic reactions in SCFs has extra advantages such as higher reaction rates with a potential of selective preparation and purification of each product.

Liu et al. (1997) synthesized cocoa butter equivalent using the enzyme-catalyzed interesterification of triglycerides (TG) high in POP (P, palmitate; O, oleate) and POO in $SCCO_2$. Among the five different lipases studied, immobilized lipase from *Mucor miehei* was the most efficient and specific. Erickson et al. (1990) investigated the effect of pressure-induced changes on the physical properties of a SCF and the rate of lipase-catalyzed transesterifications. Kamat et al. (1992; 1995) reported that $SCCO_2$ gradually inhibited *C. cylindracea* lipase. When studying the lipase-catalyzed hydrolysis of triolein in a batch system, Chi et al. (1988) reported an increase in the initial rate of the reaction with an increase in water content in both *n*-hexane and $SCCO_2$. They considered water to act as a modifier of the solvent having a one hundred fold higher solubility in $SCCO_2$ than in *n*-hexane. Glowacz et al. (1996)

studied the hydrolysis of triolein and its partial glycerides in a batch reactor system by using porcine pancreas lipase in $SCCO_2$. It was shown that the enzyme activity and the enantiomeric ratios of the products were dependent on the water content of the enzyme, substrate and the reaction time.

The literature lacks information on the hydrolysis of vegetable oils in a SCF. Therefore, with canola oil being the most important vegetable oil in Canada, the objective of this study was to investigate the effect of pressure, temperature and CO_2 flow rate on the conversion rate and product composition in the lipase-catalyzed hydrolysis of canola oil in $SCCO_2$ using a continuous flow reactor.

2.2. MATERIALS AND METHODS

2.2.1. Materials

Mucor miehei lipase immobilized on macroporous anion exchange resin, Liposyme IM, was kindly provided by Novo Nordisk (Franklinton, NC). The following information was provided by the manufacturer regarding the immobilization of Lipozyme IM and its properties. The enzyme was strongly bound on the resin by adsorption. The resin is of phenolic type and no crosslinking agents were used. The granular Lipozyme IM has a particle size of 0.2-0.6 mm. Water content of the commercial product is 2-3% (w/w). However, the optimum water content for the maximal activity of the enzyme is 10% (w/w). The enzyme is classified in the category of triacylglycerol hydrolases. It can be used in the temperature range 30-70°C. Lipozyme IM can maintain its declared activity for 1 year if it is stored at 5°C. However, at 25°C, it can retain that activity for 3 months only. Enzyme activity was provided by Novo Nordisk (Franklinton, NC) based on acidolysis of high oleic

sunflower oil with decanoic acid at 70°C for 60 min (5-6 BAUN/g of immobilized enzyme, Batch Acidolysis Units Novo). Enzyme was stored at 5°C until used in the experiments.

Canola oil was obtained from a local grocer. All lipid standards were purchased from Sigma Chemical Co. (Oakville, ON) with purities of ≥99%. Diethyl ether, HPLC grade (99.9%) and hexadecane (99%) were obtained from Aldrich Chemical Co. (Milwaukee, WI); petroleum ether, Optima from Fisher Scientific (Fair Lawn, NJ); CO_2 as reaction medium, bone dry (99.8%); hydrogen, nitrogen and helium, ultra high purity (UHP) grade (99.999%) and air, United States Pharmacopoeia (USP) grade (19.5-23.5% O_2), from Praxair (Mississauga, ON) and CO_2 as mobile phase in supercritical fluid chromatography (SFC), SFC/SFE grade (99.9999%), was from Air Products (Allentown, PA).

2.2.2. Experimental set up and design

A laboratory scale supercritical extraction system (Newport Scientific, Inc., Jessup, MD) was modified to conduct continuous enzymatic hydrolysis of canola oil in $SCCO_2$ (Fig. 2.1). Water was introduced to the CO_2 stream using an HPLC pump (Gilson 305, Middleton, WI) equipped with a manometric module (Gilson 805, Middleton, WI) at a rate of 0.004 mL/min. Oil was introduced by another HPLC pump (Varian 2010, Walnut Creek, CA) at a rate of 0.02 mL/min. A small stainless steel reaction cell (15 cm × 13 mm I.D.) was made and inserted into the original extraction cell. To ensure the uniformity of the mixture, water, oil and CO_2 were combined and passed through a mixer prior to entry at the bottom of the reaction cell. CO_2 flow rate was maintained at 3.7 L/min, measured at ambient conditions, except where the flow rate effect was studied at 1.02±0.02 and 3.71±0.10 L/min, in which

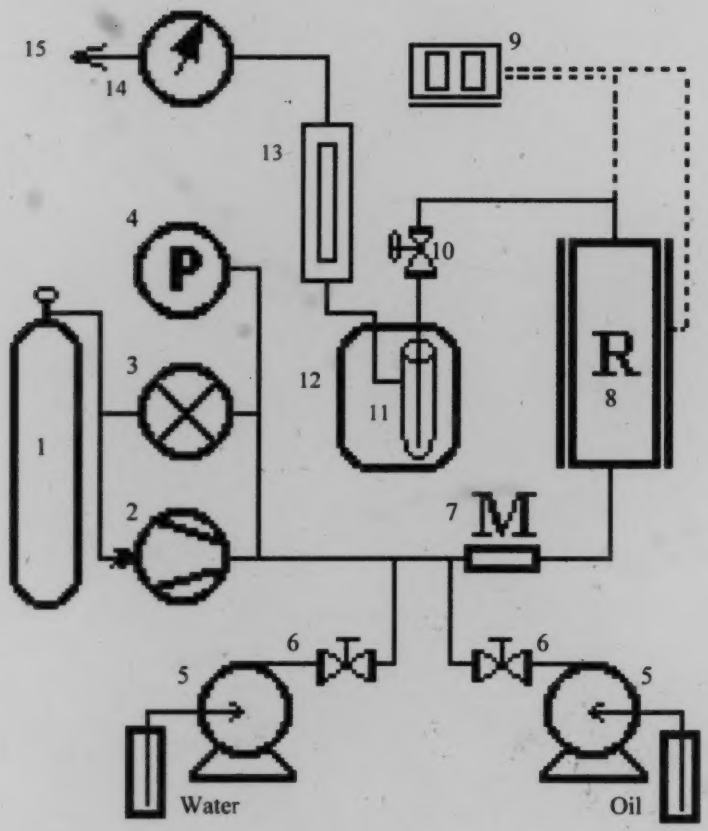

Figure 2.1. Schematic of the supercritical reaction system.
1- CO_2 tank, 2- Compressor, 3- Back pressure regulator, 4- Pressure gauge, 5- HPLC pumps, 6- On/off valve, 7- Mixer, 8- Reaction cell, 9- Temperature control, 10- Depressurization valve, 11- Sample collection tube, 12- Cold bath, 13- Rotameter, 14- Gas meter, 15- Vent.

case the averages of two runs were reported. Reactions were carried out at 10, 24 and 38 MPa and 35 and 55°C. The immobilized enzyme beads (1.0 g) were dispersed in the top portion of the reaction cell using 7.9 g glass beads (3.76 mm average O.D.). The lower portion of the reaction cell as well as the space above the enzyme layer were filled with glass wool. A thermocouple held within the enzyme bed was used to measure the reaction temperature, which was maintained within ±2°C of the desired temperature with a temperature controller (Newport Scientific, Inc., Jessup, MD). Pressure was maintained by a back-pressure regulator.

The system was thoroughly cleaned prior to each run and operated as follows: the reaction chamber was loaded with the enzyme-packed cell. All control valves were shut-off. The system was filled with CO_2 and pressurized to the desired level. The heat control system was turned on and the desired temperature was set. Water and oil pumps were initialized by pressurizing the respective lines and setting the flow rates. When the set pressures and temperatures were reached, the reaction was initiated by turning on the on/off valves in the water and oil lines as well as the depressurization valve and product flow was started. The CO_2 flow rate was adjusted with the depressurization valve. Reaction product samples were collected in two successive side-armed test tubes held in a cold bath at -20°C over the collection period (0.5-2 h), weighed and dissolved in a 1:1 (v/v) mixture of diethyl ether and petroleum ether for analysis. For each run, a new batch of enzyme was used except where the enzyme stability was studied. In addition, a blank run with the same water and oil flow rates as the enzymatic runs was conducted with no enzyme at each pressure and temperature condition studied. Although all samples were collected and analyzed, only samples at 4 h or later were selected for pattern analysis. This was necessary for the product

composition to stabilize under steady CO_2, oil and water flow rates at constant temperature and pressure.

2.2.3. Enzyme stability

Enzyme stability was examined by following the above procedure except that the same enzyme batch was used for three consecutive runs of 8 h each. The system was depressurized after each run but the enzyme was left in the reaction cell at room temperature. Due to the time needed for sample analysis and preparation procedures for the next run, there were a 5- and a 2-day time lapse in between the runs. Any leakage of the enzyme from the reactor was not expected throughout the runs and depressurization steps since the enzyme is strongly bound to the surface of the anionic exchange resin as indicated by the manufacturer.

2.2.4. Analysis of reaction products

An SFC system (SFC/GC Series 600, Dionex, Mississauga, ON) equipped with a fused silica column (10 m × 50 μm I.D.) with 0.25 μm film (SB-100-methyl silicone) and a timed-split injector was used to analyze the reaction products. The rotor capacity in the injector was 500 nL. Injection time was varied between 0.5-4.0 s to obtain a consistent response since the amount of material collected at the various conditions of the study was different. A frit restrictor (28 cm × 50 μm I.D.) at the end of the column controlled the back-pressure of the system. A flame-ionization detector (FID) at 350°C was used as the detection system. $SCCO_2$ was the mobile phase. Hydrogen and air at 0.345 MPa (50 psi) were used for flame. Nitrogen at 0.414 MPa (60 psi) was used as make-up gas. The system was run for at least 2 h prior to sample injection. Column temperature and pressure programming described by Temelli et al. (1996) was modified for the analysis. Column temperature was held at 100°C for 2

min and then increased to 190°C at a rate of 40°C/min. The program for column pressure was as follows: held at 12.2 MPa for 2 min, ramp (I) at 0.5 MPa/min to 15.2 MPa and hold for 5 min, ramp (II) at 1.5 MPa/min to 17.2 MPa hold for 3 min, ramp (III) at 1.5 MPa/min to 22.8 MPa hold for 1 min, ramp (IV) at 4.1 MPa/min to 27.4 MPa hold for 3 min and ramp (V) at 4.1 MPa/min to 31.5 MPa hold for 10 min. The Dionex Al-450 Chromatography Automation Software Release 3.32 was used to collect and analyze the data. Successive runs were ≥ 1 h apart. Relative standard deviation (RSD) in a standard mixture of FFA, MG, DG and TG was determined as 4.0%, 4.5%, 2.9% and 2.5%, respectively, which was an indication of the precision of the SFC system for quantification of lipids.

Oleic acid, mono-, di- and triolein were used as standards for FFA, MG, DG and TG, respectively. Hexadecane was used as internal standard (IS). Standard solutions containing 5, 10 and 20 mg/mL of standards and IS were prepared. Triplicate injections of each standard solution were made and response of each component in the standards was normalized to its equivalent concentration in the original solution. Relative response factors of FFA, MG, DG and TG were determined by dividing the normalized responses by that of IS in each chromatogram and then averaging for the triplicate runs of each standard. Relative response factor is a quantity which can account for possible differences in the behavior of various lipid compounds during SFC analysis. The relative response factors of different species were different, which also varied slightly with concentration. However, relative response factors are independent of injection volume and solute concentrations (Annino and Villalobos, 1992). Therefore, a mean relative response factor for all concentrations of each species was used in calculations, which was 0.80±0.02,

0.81±0.02, 0.73±0.02 and 0.96±0.02, for FFA, MG, DG and TG, respectively.

For the quantification of reaction product samples, peak area counts of FFA, MG, DG and TG were divided by the relative response factors and normalized to 100% (w/w). To determine the molar ratios of FFA, MG, DG and TG, the average molecular weights of 280, 356, 617 and 880 a.m.u., based on canola oil fatty acid composition data provided by Canola Council of Canada (1994), were used, respectively.

2.2.5. Terminology

The terms *conversion* and *production* defined as follows were used to characterize the reaction: *Conversion* was used to indicate the fraction of original TG consumed in the reaction towards the formation of any of MG, DG and glycerol. To determine the conversion, moles of each of MG, DG, TG and glycerol in the product at any given time were measured and normalized to 100%, since based on the reaction stoichiometry (eqns. 1.1 –1.4) each molecule of TG can only be converted to any one of DG, MG or glycerol. Any further breakdown of the products is not anticipated in the CO_2 environment. Then, mole fraction of TG (f_{TG}) was subtracted from unity.

$$\text{Conversion} = 1\text{-}f_{TG} \qquad (2.1)$$

Production was used to quantify the formation of individual products, i.e. MG, DG, glycerol or FFA at a given time and a specific set of conditions. The relative weight of each component found in both collection tubes of each run was reported as production.

The term *enzyme performance* was used to evaluate the overall effectiveness of the enzyme based on FFA production. Since for each step in TG hydrolysis one FFA is released, the total FFA collected can be a good measure of the extent of

hydrolysis and thus the overall performance of the enzyme. A high conversion does not necessarily pertain to a higher enzyme performance, because while conversion demonstrates the unreacted fraction of TG, enzyme performance is a measure of the overall FFA released in all three steps of the reaction.

2.2.6. Glycerol quantification

Since the amount of glycerol produced in the reaction could not be measured directly with SFC analysis, its relative quantity was calculated based on overall moles of FFA produced along with the production of each of DG, MG and glycerol (eqns. 2.2-2.4).

$$TG + H_2O \xrightarrow{lipase} DG + FFA \quad (2.2)$$

$$TG + 2\,H_2O \xrightarrow{lipase} MG + 2\,FFA \quad (2.3)$$

$$TG + 3\,H_2O \xrightarrow{lipase} Glycerol + 3\,FFA \quad (2.4)$$

The equivalent moles of FFA produced for equation (2.2) ($n_{FFA,\,DG}$) is equal to the number of moles of DG present in each product mixture (n_{DG}). Therefore, $n_{FFA,DG} = n_{DG}$. Similarly, based on the stoichiometry of equations (2.3) and (2.4), the following expressions can be obtained: $n_{FFA,\,MG} = 2\,n_{MG}$ and $n_{FFA,\,Glycerol} = 3\,n_{Glycerol}$, where $n_{FFA,\,MG}$ and $n_{FFA,\,Glycerol}$ are the number of moles of FFA equivalent to the moles of MG (n_{MG}) and glycerol ($n_{Glycerol}$), respectively. Therefore, the total FFA present in the product mixture ($n_{FFA,\,total}$) can be expressed as

$$n_{FFA,\,total} = n_{FFA,\,DG} + n_{FFA,\,MG} + n_{FFA,\,Glycerol} \quad (2.5)$$

By replacing the $n_{FFA,\,DG}$, $n_{FFA,\,MG}$ and $n_{FFA,\,Glycerol}$ with n_{DG}, $2n_{MG}$ and $3n_{Glycerol}$, respectively, an expression for the total number of moles of FFA can be obtained

$$n_{FFA,\,total} = n_{DG} + 2\,n_{MG} + 3\,n_{Glycerol} \quad (2.6)$$

which can be converted to the following, since $n_{FFA,\,total}$, n_{DG} and n_{MG} are determined

through SFC analysis

$$n_{Glycerol} = (n_{FFA,\ total} - n_{DG} - 2\ n_{MG})/3 \qquad (2.7)$$

Equation 2.7 was used to determine the relative moles of glycerol.

2.3. RESULTS AND DISCUSSION

Analysis of the canola oil used as the substrate for the hydrolysis reaction showed only TG peaks and there was no evidence of the presence of MG, DG or FFA. Blank runs carried out at different pressure and temperature conditions with no enzyme indicated that hydrolysis of TG in supercritical medium was negligible at all conditions compared to those with the enzyme (Table 2.1). A typical SFC chromatogram of the reaction products is given in Figure 2.2. The retention times of FFA, MG, DG and TG were 10-16, 17-19, 23-27 and 27-31 min, respectively. The wider peaks of FFA and TG indicate that these lipid classes consist of a mixture of different components. On the other hand, the sharper peaks of MG and DG represent the narrower range of product spread within MG and DG. The fatty acid composition

Table 2.1. Product composition for blank runs (with no enzyme) based on averages of 3rd and 4th h extracts.

P (MPa)	T (°C)	Product Composition (mole%)			
		TG	DG	MG	FFA
38	55	95.2%	2.2%	0.0%	2.6%
38	35	96.2%	1.5%	0.0%	2.3%
24	55	98.1%	0.0%	0.0%	1.9%
24	35	95.5%	2.5%	0.0%	2.1%
10	35	97.5%	0.0%	0.0%	2.5%

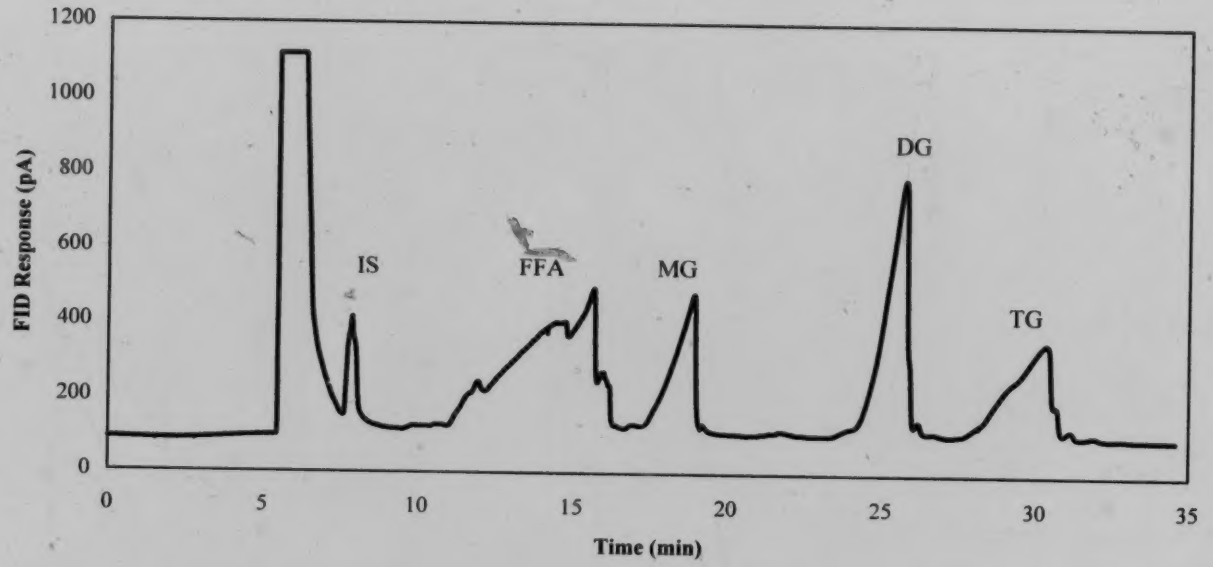

Figure 2.2. Typical supercritical fluid chromatogram of a product mixture from the enzymatic hydrolysis of canola oil in $SCCO_2$; IS, internal standard; FFA, free fatty acids, MG, monoglycerides; DG, diglycerides; TG, triglycerides.

Table 2.2. Fatty acid composition of canola oil.[a]
[FA: Fatty acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids].

Fatty acid	Composition (w/w%)
$C_{14:0}$	0.1
$C_{16:0}$	3.5
$C_{18:0}$	1.5
$C_{20:0}$	0.6
$C_{22:0}$	0.3
Total Saturated FA	6.0
$C_{16:1}$	0.2
$C_{18:1}$	60.1
$C_{20:1}$	1.4
$C_{22:1}$	0.2
Total MUFA	61.9
$C_{18:2}$	20.1
$C_{18:3}$	9.6
Total PUFA	29.7

[a] Adopted from Canola Council of Canada (1994).

of canola oil presented in Table 2.2 adopted from Canola Council of Canada (1994) demonstrate that the C_{18} fatty acids make up >90% of the total fatty acids in canola oil. It is possible that the MG and DG fractions contain C_{18} unsaturated fatty acids only thus giving a narrower peak. However, it needs to be confirmed by conducting fatty acid composition analysis of each of the FFA, MG, DG and TG fractions.

2.3.1. Enzyme stability in $SCCO_2$

Changes in the concentrations of FFA, MG, DG and TG over a 24 h period at 38 MPa and 55°C are given in Figure 2.3. A higher concentration of FFA at the start of each 8 h run is due to the extra time needed for the system to reach the desired pressure and temperature during which oil was in contact with the enzyme. The results after the 4th h of each step were considered for pattern analysis. The 2- and 5-day delays in between the runs did not seem to cause any extra loss in enzyme activity

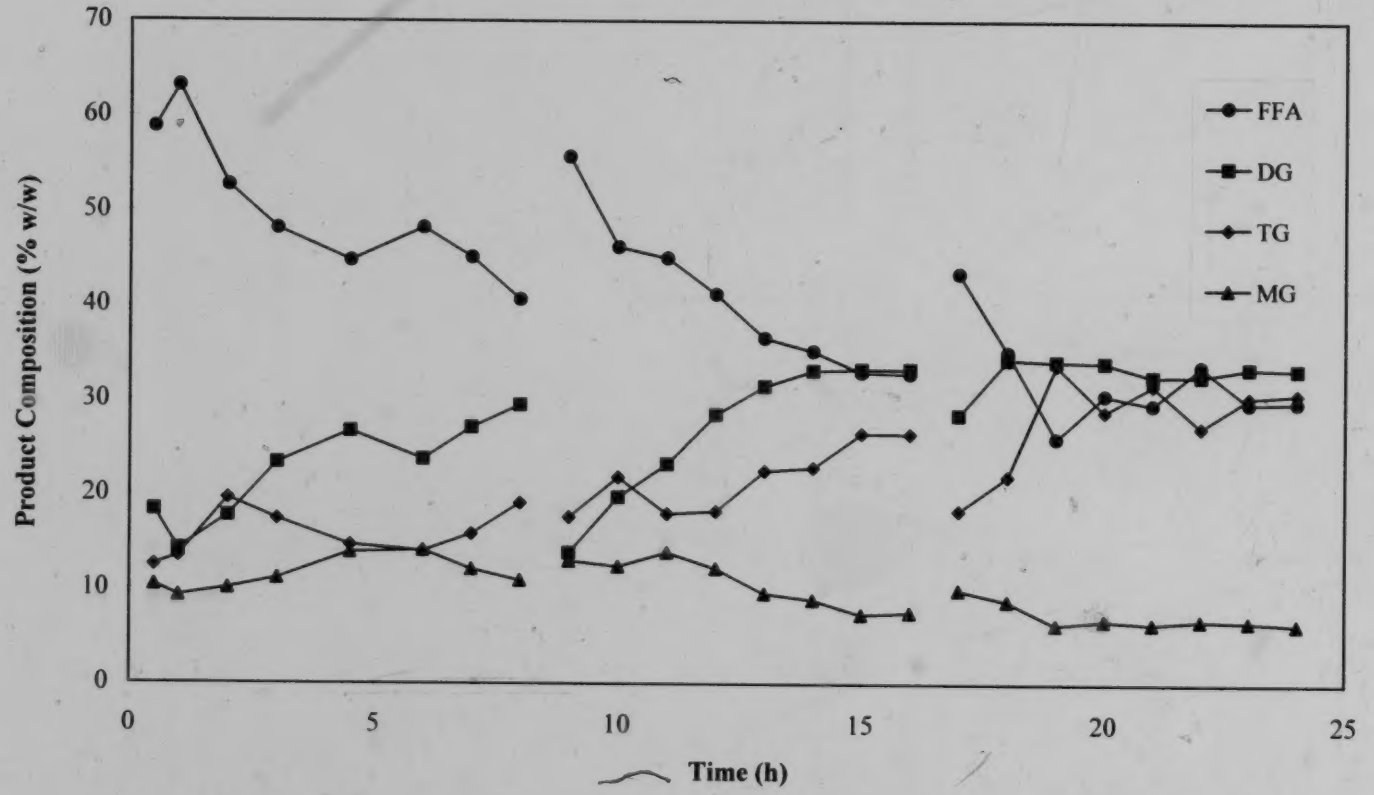

Figure 2.3. Change in the product composition in the lipase-catalyzed hydrolysis of canola oil in $SCCO_2$ at 38 MPa and 55°C.

since the product compositions followed nicely from one run to the next without a noticeable drop in between. The decline in the consumption of TG and the decrease in the production of FFA and MG indicated a loss (~34%, based on the drop in TG consumption) in enzyme activity as reaction proceeded. This is consistent with the results of Marty et al. (1992), who used *Mucor miehei* lipase immobilized on a macroporous anionic resin and reported a 10% loss in the enzyme activity in both $SCCO_2$ and *n*-hexane after 6 days of exposure to 13 MPa and 40°C. However, an increase in pressure to 18 MPa at 40°C did not affect the stability. An additional 10% loss in activity was observed by increasing the temperature to 60°C at 13 MPa. Although the extent of inactivation increases with the number of pressurization-depressurization steps (Randolph et al., 1991), its contribution to the loss of enzyme activity in this experiment is not anticipated to be significant. Giessauf et al. (1999) reported that lipase from *Pseudomonas sp.* and lipase from *Candida cylindracea* lost only 36.1% of its activity after 30 pressurization-depressurization steps in 24 h in $SCCO_2$ at 15 MPa and 75°C. In this study, only three pressurization-depressurization steps were involved and the reaction temperature (55°C) was milder than that applied by Giessauf et al. (1999). The higher pressure in this study (38 MPa) compared to a lower pressure in the study of Giessauf et al. (1999) does not contribute to the enzyme inactivation since it has been reported that such moderate pressures do not deactivate the enzyme (Lozano et al., 1996).

Different enzymes exhibit different activity patterns in supercritical media, possibly because of different immobilization support (Yu et al., 1992) and different structures. For example, *C. cylindracea* lipase supported on Celite 545 was shown to

deactivate at a higher rate than *M. miehei* lipase supported on macroporous anionic resin beads (Yu et al., 1992). At 13.6 MPa and 40°C, the residual activity of the former was 75% and that of the latter was 85-90% after 7 and 6 days, respectively (Yu et al., 1992). Chymotrypsin and trypsin can be mentioned to illustrate the structure effect on enzyme stability. These enzymes contain disulfide bridges and undergo partial inactivation as a result of slow depressurization, ~5-10 min, from 10 MPa after exposure to $SCCO_2$ medium (Randolph et al., 1991). However, penicillin amidase, which does not have cysteine, appears to be the least stable during depressurization (Randolph et al., 1991). Giessauf et al. (1999) reported that hydrolases (lipases and esterases, crude preparations) with disulfide bridges had a lower degree of inactivation compared to an enzyme without cystine after several pressurization-depressurization steps at 15 MPa and 75°C for 24 h. However, thermal stability was not any better. Zagrobelny and Bright (1992) reported that the inactivation could also take place during pressurization. How Lipozyme IM was affected in this study needs further research.

A higher DG content of the product is a demonstration of low extent of hydrolysis since DG is the first intermediate product of the TG hydrolysis. On the other hand, a higher MG content along with a lower DG content can indicate a more efficient reaction. Similar conclusion applies if a lower TG content and/or an absence of MG and DG along with a high FFA content are observed.

2.3.2. Effect of CO_2 flow rate on product composition

Effect of CO_2 flow rate on the hydrolysis of canola oil in $SCCO_2$ was studied at 38 MPa and 55°C applying two different CO_2 flow rates of 1.02 ± 0.02 and 3.71 ±

0.10 L/min (measured at ambient conditions) over 8 h. Figure 2.4 depicts the changes in glycerol content of the product at the two different flow conditions. The fluctuations in the data within the first two hours demonstrate the instability in the product composition early in the reaction. The mole fractions of MG and DG were lower at lower flow rate indicating further conversion to glycerol. This is evident in the higher fraction of glycerol in the final product at this condition. Thus, the enzyme is capable of fully hydrolyzing TG molecules if enough residence time is provided. TG content of the product mixtures was higher at lower flow rate. This result along with those obtained for MG and DG demonstrated that there was a parallel conversion of MG and DG to glycerol after a certain amount of MG and DG was produced. This was verified by plotting the total FFA production at the two different flow rates for the last 4 h of the reaction where product composition was stabilized (Fig. 2.5). It was demonstrated that there was no flow rate effect on the overall enzyme performance since the FFA production was not affected by a change in the CO_2 flow rate.

The total amount of materials collected at the end of each experiment was about 67% of the sum of oil and water introduced to the system. The remaining 33% consists of: i) some unused water which was collected in the reaction chamber after the reaction was over, ii) some oil which was swept away during the depressurization of the system, and finally iii) some oil and water which was left in the connecting tubes prior to the reactor.

The solubility of canola oil at different conditions was calculated from the slope of the linear portion of extraction curves by Fattori et al. (1988). The solubility of canola oil in $SCCO_2$ at 36 MPa and 55°C is 11 mg oil/g CO_2 (Fattori et al., 1988),

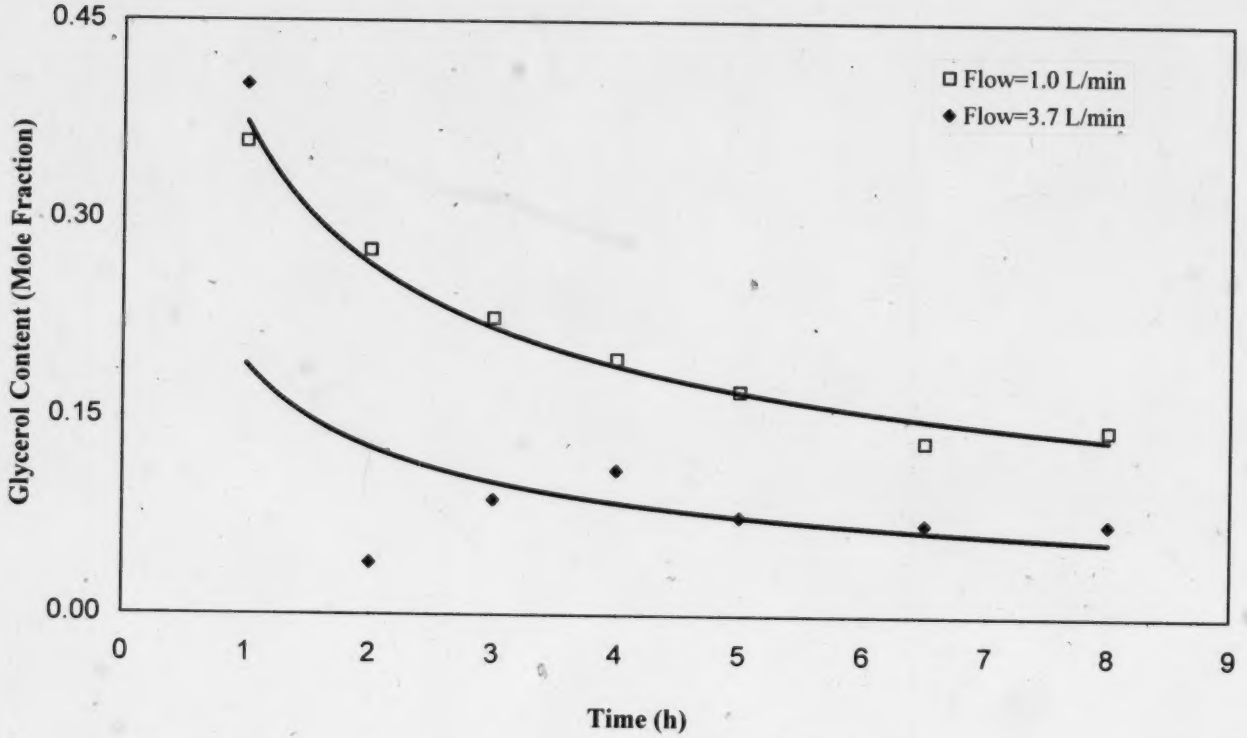

Figure 2.4. Flow effect on glycerol content in the continuous hydrolysis of canola oil in $SCCO_2$ at 38 MPa and 55°C.

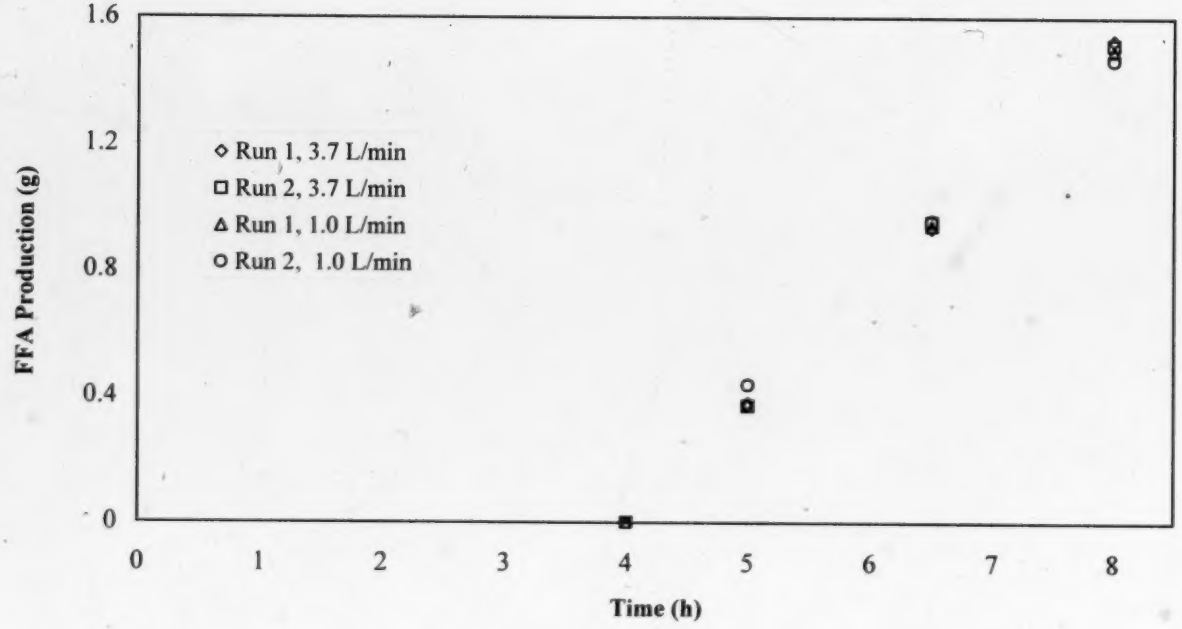

Figure 2.5. Effect of CO_2 flow rate on the FFA production after 4 h of reaction at 38 MPa and 55°C.

which is expected to be slightly higher at 38 MPa and 55°C, i.e. conditions of this study. Also, the solubility of water in $SCCO_2$ at 55°C and 40.5 MPa is ~7 mg water/g CO_2 (Wiebe and Gaddy, 1941), which is approximately the same condition of our study. Therefore, up to a total of ~40 g oil and ~25 g water at 3.71 L/min CO_2 flow rate and ~11 g oil and ~7 g water at 1.02 L/min CO_2 flow rate would have been dissolved over 8 h if they were supplied in sufficient quantities to the CO_2 stream. The flow rates of oil and water (0.02 and 0.004 mL/min, respectively) were kept constant throughout this study providing 9.89 and 1.92 g oil and water over 8 h, respectively.

At a higher CO_2 flow rate the concentration of the substrates in the CO_2 phase is below saturation since the same amount of oil and water was supplied to a larger CO_2 quantity (~440 g) compared to that of the lower flow rate (~120 g) over 8h. Whether the supercritical medium is saturated or not has an effect on the hydrolysis reaction. The lower concentration of substrates in the reactor as a result of a higher CO_2 flow rate may cause the reaction rate to drop accordingly. Thus, the condition is not appropriate for complete hydrolysis of TG molecules to glycerol and instead partial conversion to the intermediate products is favored. Glowacz et al. (1996) showed that the extent of hydrolysis of triolein and its partial glycerides increased with reaction time and as a result a higher amount of oleic acid was released.

Another effect may be related to the possible loss of enzyme activity as a result of a lack of water around the enzyme at the higher CO_2 flow rate. The layer of water on the enzyme has more tendency to leave the enzyme site at the higher CO_2 flow rate since water concentration in the SCF is lower. Hampson and Foglia (1999) reported that the immobilized lipase from *Candida antarctica* lost 2-6% of its water

content per hour in $SCCO_2$ at 27 MPa and 60°C with 0.5 or 1 L/min CO_2 flow rate. They also reported that in the hydrolysis of tripalmitin, the enzyme with 1.5% moisture content gave little evidence of hydrolysis. However, the enzyme with 5.4-23.5% initial moisture content led to products with palmitic acid and unreacted tripalmitin only, which is in agreement with the results of the lower flow rate in this study.

Since the amounts of oil and water introduced to the reaction system were below their saturation levels in $SCCO_2$, the results obtained in this study can be attributed to the net flow rate effect on the hydrolysis reaction. It seems that product composition can be controlled by changing flow rates of substrates and CO_2. Furthermore, one can predict that full hydrolysis of oil is possible if a lower CO_2 flow rate, a higher enzyme load and/or a lower oil flow rate is selected. On the other hand, a higher production of MG can be achieved if a higher CO_2 flow rate, a lower enzyme load and/or a higher oil flow rate (up to the saturation level of $SCCO_2$ at the conditions of study) is selected.

2.3.3. Composition of the product mixtures

A typical trend for the change in product composition over a 4 h period is shown in Figure 2.6, for a reaction at 24 MPa and 35°C. It is apparent that the reaction system requires about 3 h to reach steady state. After 4 h, DG concentration (~20% mole/mole) was twice that of MG (~10% mole/mole) indicating that DG production was favored over MG at this condition. Similar results were obtained for all other conditions of this study except for the reactions conducted at 10 MPa and 35°C, in which case the production of both MG and DG was less pronounced. At 10 MPa and 35°C, due to the poor solubility of canola oil in $SCCO_2$ (0.05 mg oil/g CO_2,

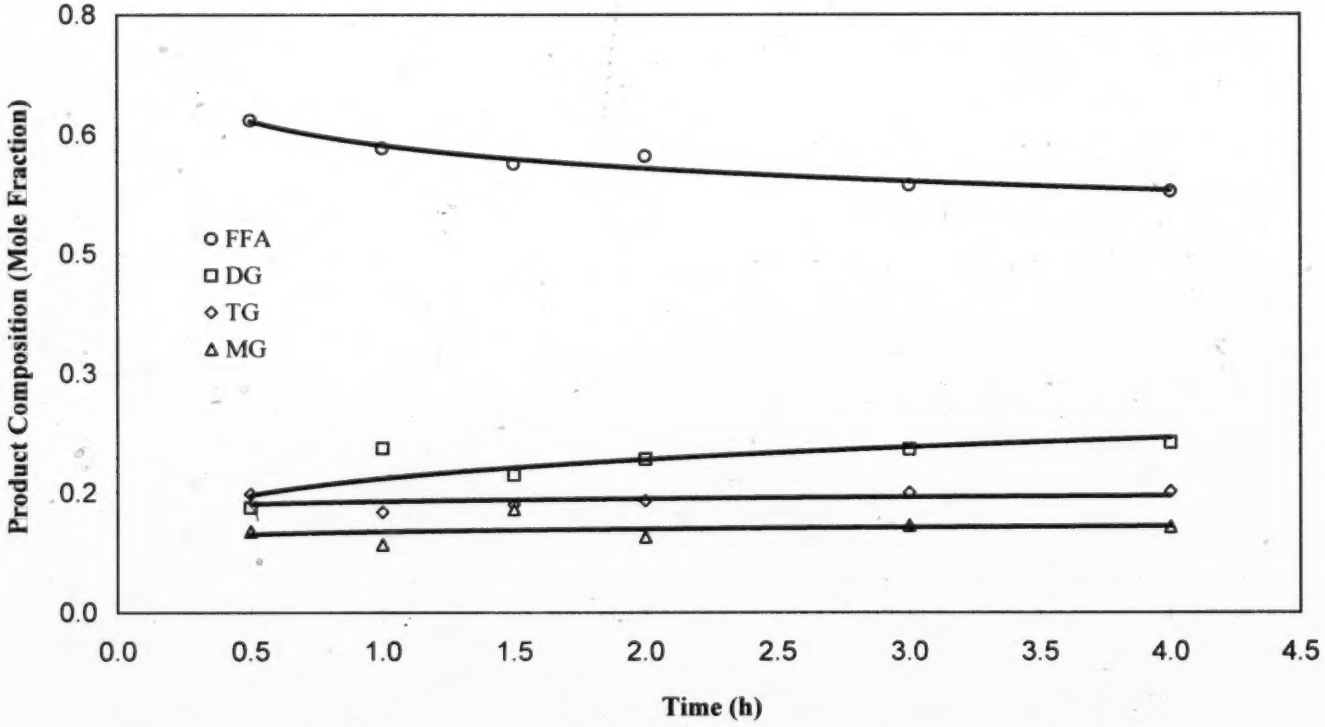

Figure 2.6. Change in the product composition in the hydrolysis of canola oil in $SCCO_2$ at 24 MPa and 35°C (CO_2 flow rate: 3.7 L/min, measured at ambient conditions).

Fattori et al., 1988) only a small fraction (0.5%) of pumped oil was dissolved while water solubility (~3 mg water/g CO_2) was relatively high (Wiebe and Gaddy, 1941) and as a result, enzyme was able to fully hydrolyze most of the TG available to the catalytic sites. Therefore, the accumulation of the intermediate products, MG and DG, was not favored and complete hydrolysis was achieved. When Glowacz et al. (1996) studied the hydrolysis of triolein and its partial glycerides, they reported that the (re)activity of triolein was higher than dioleins and within the dioleins, 1,2-diolein was more (re)active than 1,3-diolein and finally dioleins were more (re)active than 1-monoolein indicating compound specificity of the immobilized porcine pancreatic lipase. Water content of the immobilized lipase was also an important factor in the hydrolytic activity of the enzyme.

2.3.4. Effect of temperature and pressure on product composition

Relative concentrations of glycerol, MG, DG and TG in the product mixture after 4 h of reaction are listed in Table 2.3 for different pressures and temperatures of this study. The concentration of each component in the product mixture was mainly dependent on pressure and small differences were observed with a change in temperature at each pressure. Glycerol concentration was highest (~35 mole %) at 10 MPa and 35°C. This was related to the lower solubility of oil in $SCCO_2$ at this condition resulting in further hydrolysis. At 38 MPa, glycerol content increased with an increase in temperature from 35 to 55°C. The highest level of MG was obtained at 24 MPa and 35-55°C, which was declined by an increase in pressure to 38 MPa. At 10 MPa and 35°C, concentrations of both MG and DG were low indicating their immediate consumption on the enzyme site. DG content increased dramatically with a

Table 2.3. Effect of pressure and temperature on the composition of glycerol, MG, DG and TG normalized to 100% (mole %) after 4 h of continuous reaction.

Compound	10 MPa/35°C	24 MPa/35°C	24 MPa/55°C	38 MPa/35°C	38 MPa/55°C
Glycerol	34.5%	5.8%	3.5%	6.9%	9.7%
MG	1.3%	21.4 %	20.9%	14.4%	13.4%
DG	15.1%	39.5%	41.4%	42.8%	39.7%
TG	49.1%	33.3%	34.1%	35.9%	37.3%

change in the properties of the microaqueous layer of the enzyme or a reduction in the mass transport properties of the solvent can also be a factor. When studying the acyltransfer activity, Briand et al. (1995) showed that the acyltransfer (including hydrolysis) activity of *Candida parapsilosis* lipase was very sensitive to the experimental temperature and an optimum temperature of 40-50°C was obtained in an aqueous treatment of rapeseed oil. They further demonstrated that the enzyme was able to maintain its activity at temperatures up to 50°C but substantially inactivated at temperatures >50°C in less than an hour in aqueous solutions. Similarly, they observed very high sensitivity of the enzyme activity to a change in the pH and the concentration of entrainers such as methanol and surfactants like sodium dodecyl sulfate and poly(vinyl)alcohol.

2.3.5. Effect of pressure and temperature on the production

The amount of MG, DG and FFA in the product mixture was highest at 24 MPa (Figs. 2.7-2.9, respectively). The highest production of MG was obtained at 24 MPa and 35°C, after which an increase in both pressure and temperature or a decrease in pressure resulted in an adverse effect on the production of MG. The pressure and temperature effect on the production of DG was less pronounced than that on MG production. Temperature effect on the FFA production at 35-55°C and 24-38 MPa

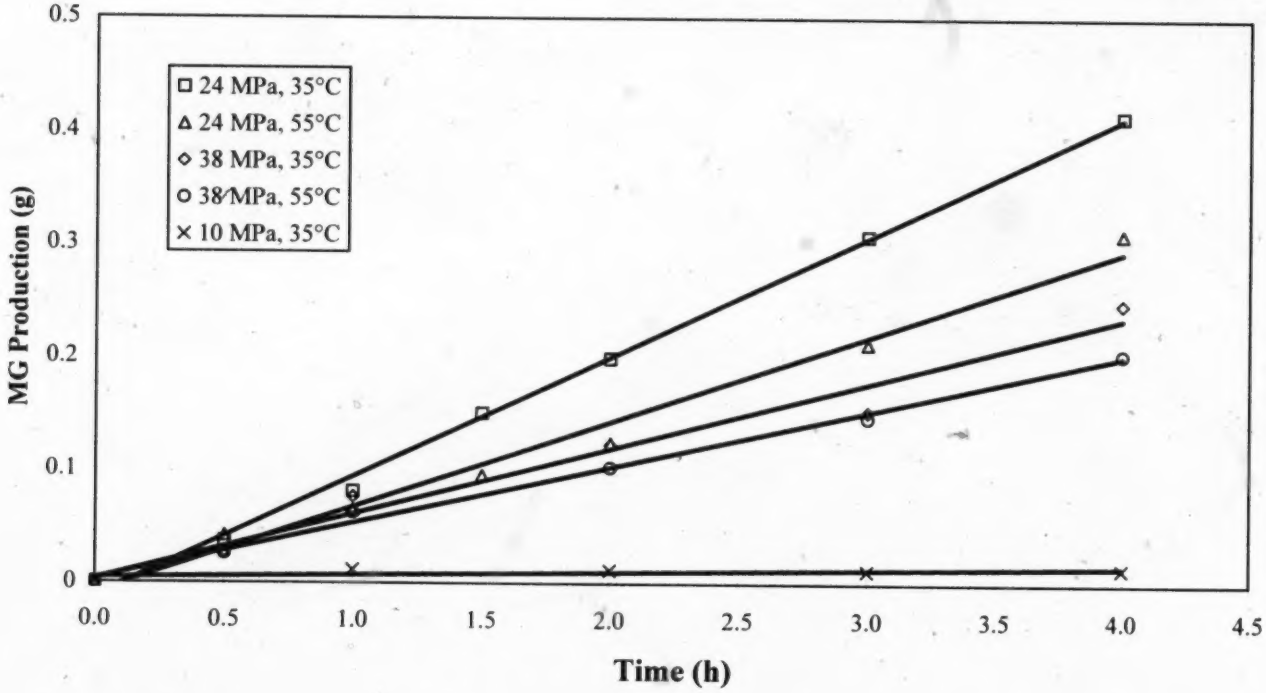

Figure 2.7. Effect of pressure and temperature on MG production in $SCCO_2$ (CO_2 flow rate: 3.7 L/min, measured at ambient conditions).

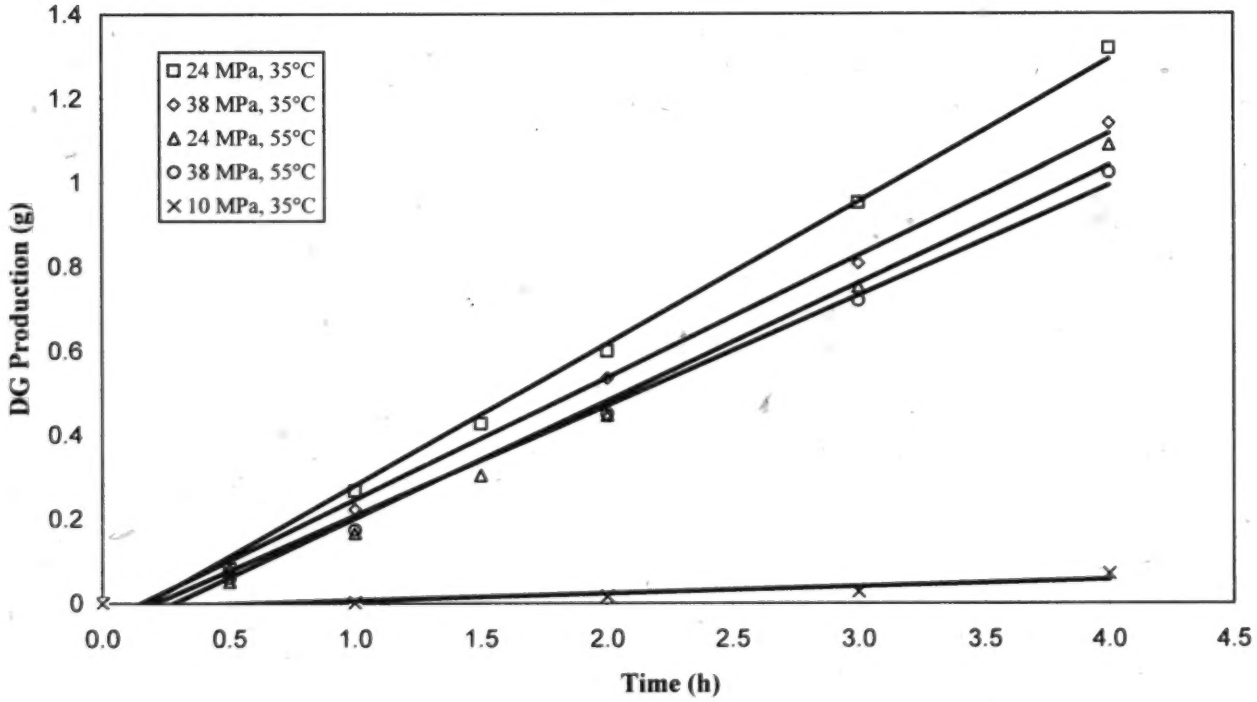

Figure 2.8. Effect of pressure and temperature on DG production (CO_2 flow rate: 3.7 L/min, measured at ambient conditions).

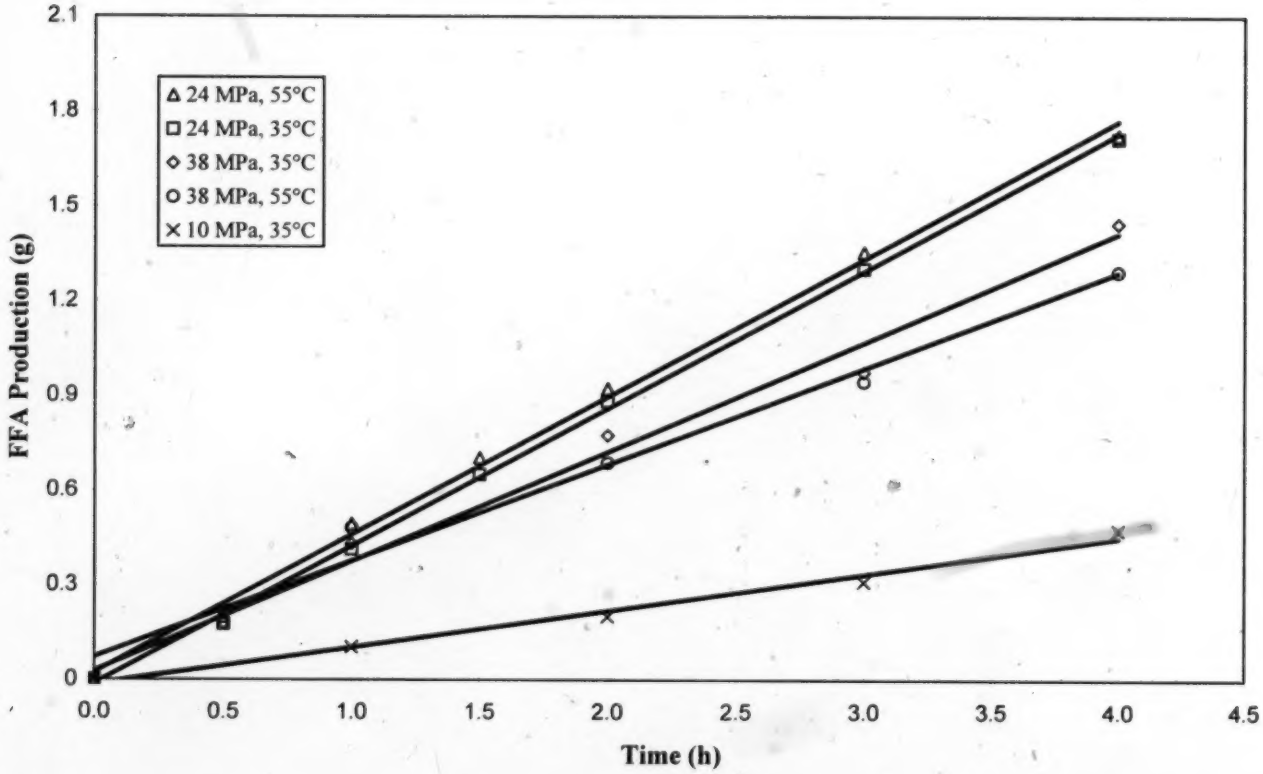

Figure 2.9. Effect of pressure and temperature on FFA production (CO_2 flow rate: 3.7 L/min, measured at ambient conditions).

was not pronounced, but pressure was an important factor. At this range of pressure and temperature, the total oil load of CO_2 does not change since the total oil introduced to the system was lower than the saturation level of CO_2. FFA production was higher at 24 MPa than that at 38 and 10 MPa. At 10 MPa, the lowest production of MG, DG and FFA was obtained, which could be related to the lower solubility of canola oil in $SCCO_2$ at this condition (0.05 mg oil/g CO_2) compared to that of other conditions (5-11 mg oil/g CO_2) (Fattori et al., 1988).

2.4. CONCLUSIONS

Immobilized lipase, Lipozyme IM, showed a different performance at various pressure and temperature conditions and as a result the production of MG, DG and glycerol was affected by a change in pressure and temperature over 10-38 MPa and 35-55°C, respectively. Enzyme performance was highest at 24 MPa and 35°C. The solubility of oil in $SCCO_2$ was low at 10 MPa and 35°C and as a result the total product collected was much less than those of other conditions. However, within the amount solubilized, the accumulation of intermediate products, MG and DG, was not favored and more of final product, glycerol, was formed. Highest conversion as well as highest MG and DG production was obtained at 24-38 MPa and 35-55°C. There was a loss in the enzyme activity at 38 MPa and 55°C over a 24 h reaction period.

Although at a constant flow rate of oil and water a change in the CO_2 flow rate did not influence the enzyme performance, the product composition was affected. By decreasing the flow rate of CO_2, full hydrolysis of canola oil can be achieved. Similarly, it is predicted that an increase in the enzyme load and/or a decrease in the

quantity of the oil substrate can promote the full hydrolysis of oil. Lipase-catalyzed hydrolysis of canola oil in $SCCO_2$ show potential for conversion of fats and oils to higher value products such as MG, DG, FFA and glycerol. Pressure, temperature and CO_2 flow rate are important parameters in controlling the composition of the product mixture.

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3. ON-LINE EXTRACTION-REACTION OF CANOLA OIL USING IMMOBILIZED LIPASE IN SUPERCRITICAL CO_2[1]

3.1. INTRODUCTION

Feasibility of conducting enzymatic reactions in supercritical fluids (SCFs) encourages research activity involving extraction, reaction, fractionation and analysis of lipids in such media. The ease of manipulating the properties of SCFs has promoted their use as a medium to conduct enzymatic reactions at a high rate. On-line extraction-fractionation of lipid components has been reported (Reverchon, 1997). However, extraction of food materials followed by their on-line reaction and post-reactional separation of the reaction products has not been investigated even though it shows great potential for the food industry.

As a benign and affordable solvent, supercritical CO_2 ($SCCO_2$) has been commonly used as a solvent for the extraction processes both at the research level and in the industrial and pilot-plant scale such as extraction of spices and flavors and decaffeination of coffee. Enzymatic lipid reactions such as hydrolysis, (inter)esterification, alcoholysis and acidolysis have been studied in $SCCO_2$ environment. Synthesis of cocoa butter equivalent (Liu et al., 1997), hydrolysis of triolein (Chi et al., 1988; Glowacz et al, 1996), hydrolysis of tripalmitin (Hampson and Foglia, 1999) and transesterification of triglycerides (TG) (Erickson et al., 1990) are examples of enzyme-catalyzed lipid reactions carried out in $SCCO_2$.

Lipase-catalyzed hydrolysis of canola oil in $SCCO_2$ shows potential for

[1] A version of this chapter is to be submitted to the Journal of Supercritical Fluids for consideration for publication.

conversion of fats and oils to higher value products such as mono- and diglycerides (MG and DG, respectively), glycerol and free fatty acids (FFA). The stepwise hydrolysis of TG to DG, MG, glycerol and FFA was described in section 1.1.2. Depending on the extent of hydrolysis, a mixture of TG, DG, MG, glycerol and FFA can be obtained in the product mixture. The effect of pressure, temperature and CO_2 flow rate on the hydrolysis of canola oil in $SCCO_2$ using immobilized lipase from *Mucor miehei* was discussed in chapter 2. Production of MG and DG was influenced by a change in pressure and temperature over 10-38 MPa and 35-55°C, respectively. Product composition was also affected by a change in CO_2 flow rate at a constant flow rate of oil and water.

For the enzymatic hydrolysis of canola oil described above, previously refined canola oil was used as thesubstrate. Conventional extraction and refining technology for edible oils is costly and involves many steps including seed cleaning, preheating, crushing/flaking, cooking, pressing, solvent extraction, oil desolventizing, degumming, acid treatment, bleaching or alkali refining, dewaxing, and physical refining/deodorization (Canola Council of Canada, 1994). Furthermore, the use of *n*-hexane, a flammable and environmentally hazardous organic solvent, as the oil extraction solvent is being restricted and it is anticipated that *n*-hexane will be banned in many extraction and analytical applications in the near future. With the latest developments in the supercritical fluid extraction (SFE) technology and expansion of its industrial applications to food products such as spices, flavors and coffee (Stahl et al., 1988; Reverchon, 1997), SFE is gaining importance as an alternative to the

conventional methods of oil extraction. Furthermore, the traditional enzyme-catalyzed reactions in aqueous media as well as those performed in organic solvents will face strong competition from the ever-growing SCF technology. With such developments in both extraction and reaction processes, the convenience of on-line reaction of the extracted materials will merge these two technologies to avoid several unnecessary steps involved in between the independent extraction and reaction processes.

Such an integrated approach is a possibility that is presently in its earliest stages of development. Small scale on-line supercritical fluid extraction/supercritical fluid reaction (SFE/SFR) and on-line SFE/SFR/gas chromatography (GC) were reported by Jackson and King (1996) and Snyder et al. (1996), respectively, for soybean oil. However, they incorporated both extraction and reaction processes in a single cell where it was not possible to apply separate operating conditions for each of these processes. There are no reports in the literature on an on-line extraction/reaction process with separate extraction and reaction chambers equipped with separate controls on pressure, temperature and moisture content. Developing such an on-line system will eliminate some processing steps in between and as a result will save time, energy and other resources. As well, such an approach can facilitate the control of the feed amount to the reaction system by only changing the operational conditions of the extraction process and can provide an opportunity to use the phenomena involved in an extraction process in operating a reaction system. Therefore, the objectives of this study were: a) to develop an on-line SFE/SFR system to sequentially extract the oil from canola flakes and enzymatically hydrolyze it in $SCCO_2$, and b) to investigate the effect of CO_2 flow rate, enzyme load and the quantity of canola flakes on the composition of hydrolysis products.

3.2. MATERIALS AND METHODS

3.2.1. Materials

Cooked canola flakes, *Brassica napus* and *B. campestris*, and *Mucor miehei* lipase immobilized on macroporous anionic exchange resin, Liposyme IM, were kindly provided by Canamera Foods (Fort Saskatchewan, AB) and Novo Nordisk (Franklinton, NC), respectively. Enzyme was stored at 5°C until used in the experiments. Enzyme activity was provided by Novo Nordisk (Franklinton, NC) based on acidolysis of high oleic sunflower oil with decanoic acid at 70°C for 60 min (5-6 BAUN/g of immobilized enzyme, Batch Acidolysis Units Novo). All lipid standards were purchased from Sigma Chemical Co. (Oakville, ON) with purities of ≥99%. Diethyl ether, HPLC grade (99.9%) and hexadecane (99%) were obtained from Aldrich Chemical Co. (Milwaukee, WI); petroleum ether, Optima from Fisher Scientific (Fair Lawn, NJ); chloroform and methanol, Assurance grade (99.8%), isopropanol (99.9%) and hexane (85% *n*-hexane), OmniSolv grade from BDH, Inc. (Toronto, ON); CO_2 as reaction medium, bone dry (99.8%); hydrogen, nitrogen and helium, ultra high purity (UHP) grade (99.999%) and air, United States Pharmacopoeia (USP) grade (19.5-23.5% O_2), from Praxair (Mississauga, ON) and CO_2 as carrier in the supercritical fluid chromatography (SFC), SFC/SFE grade (99.9999%), from Air Products (Allentown, PA).

3.2.2. Experimental setup and design

A laboratory scale supercritical extraction system (Newport Scientific, Inc., Jessup, MD) was modified to conduct continuous extraction and hydrolysis of canola oil in $SCCO_2$ (Fig. 3.1). A new stainless steel extraction cell (13 cm × 29 mm I.D.)

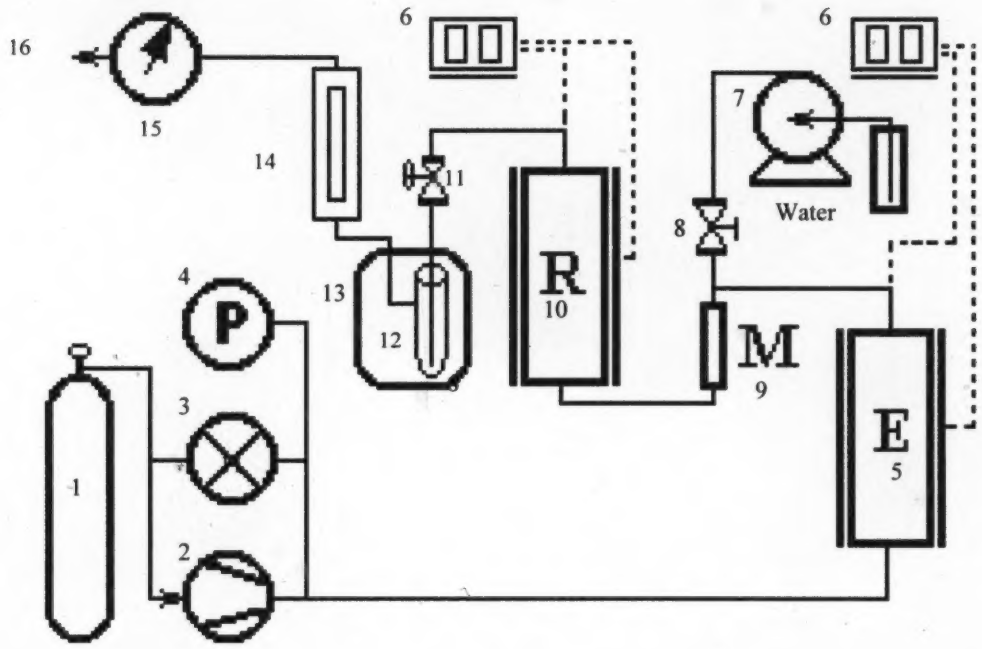

Figure 3.1. Schematic of the supercritical extraction-reaction system.
1- CO_2 tank, 2- Compressor, 3- Back pressure regulator, 4- Pressure gauge, 5- Extraction chamber, 6- Temperature control, 7- HPLC pump, 8- On/off valve, 9- Mixer, 10- Reaction cell, 11- Depressurization valve, 12- Sample collection tube, 13- Cold bath, 14- Rotameter, 15- Gas meter, 16- Vent.

was fabricated and positioned after the compressor. Water was pumped in using a piston pump (Gilson 305, Middleton, WI) equipped with a manometric module (Gilson 805, Middleton, WI) at a rate of 0.004 mL/min and mixed with oil-laden CO_2 exiting the extractor. A mixer was located close to the bottom of the reaction cell, where oil-laden CO_2 and water were mixed prior to entry into the reactor. The CO_2 flow rate was 0.5 L/min, measured at ambient conditions, except where the flow rate effect was studied at 0.5 and 3.9 L/min, in which case the averages of duplicate runs were reported. The flow rates chosen were close to the minimum and maximum limits of the compressor. Reactions were carried out at 24 MPa and 35°C. A small stainless steel reaction cell (15 cm ×13 mm I.D.) was made and inserted into the original extraction cell. Immobilized enzyme beads were mixed with glass beads (7.9 g, 3.76 mm average O.D.) to prevent formation of a compact enzyme bed. The effect of enzyme load was investigated by using different amounts of enzyme, 1.0, 2.0 and 5.0 g, which were mixed with 7.9, 15.8 and 2.7 g glass beads, respectively. The quantity of glass beads was adjusted for each enzyme load based on the total reactor volume. Enzyme and glass bead mixture was placed in the top portion of the reaction cell. The lower portion of the cell as well as the space above the enzyme layer were filled with glass wool. The extraction and reaction temperatures were measured using two separate thermocouples held within the top portions of the respective chambers, and maintained within ±2°C of desired temperatures with separate controllers (Milton Roy, Ivyland, PA, and Newport Scientific, Inc., Jessup, MD, respectively). Pressure was maintained by a back-pressure regulator.

The system was cleaned thoroughly prior to each run and operated as follows:

Canola flakes and enzyme-packed cell were loaded into the extraction and reaction chambers, respectively. All control valves were shut off. The system as filled with CO_2 and pressurized to the desired level. The heat control system was turned on and the desired temperature was set. Water pump was initialized by pressurizing the water line and setting the flow level. When all the system pressures and temperatures reached the set levels, reaction was initiated by turning on the on/off valve in the water line as well as the deppressurization valve and adjusting the CO_2 flow rate. Reaction product samples were collected into two successive side-armed test tubes held in a cold bath at -20°C and products were collected every hour, weighed and dissolved in a 1:1 (v/v) mixture of diethyl ether and petroleum ether for analysis. For each run, a new batch of enzyme was used. For each enzymatic reaction, a blank extraction run was conducted with no enzyme at the same pressure, temperature, water and CO_2 flow rates, and amount of canola flakes. Except for the blank runs, the results reported for each condition are means of duplicate runs.

3.2.3. Determination of moisture, FFA and total lipid contents of canola flakes

The moisture content of canola flakes before and after the extraction-reaction process was determined using AOAC method 925.10 (AOAC, 1990). The FFA content of the original canola flakes was analyzed according to Ke and Woyewoda (1978). However, the Soxhlet extracted oil samples (in *n*-hexane) were used as the starting material. In addition, a pH-meter was used along with the indicator *m*-cresol to determine the end point.

The official method Ba 3-38 based on Soxhlet extraction (AOCS, 1954) was used to determine the oil content of the original canola flakes, except that a total of 4 g

canola flakes was extracted using *n*-hexane. Solvent removal from the extract was carried out using a low-pressure rotary evaporator at ≤40°C instead of the direct heating suggested in the method.

3.2.4. Analysis of reaction products

Product samples were analyzed by supercritical fluid chromatography according to the procedure described in section 2.2.4. The only difference was that in this series of analysis, the injection times were varied from 0.5 to 4.0 sec.

3.2.5. Terminology

The terms *production* and *enzyme performance* were defined similar to those given in section 2.2.5.

3.3. RESULTS AND DISCUSSION

The analysis of the cooked canola flakes as obtained from a local processor indicated 7.0% (w/w) moisture and 43.5% (w/w) oil content. Moisture content of the flake residues after $SCCO_2$ extraction was within the range 3.7-5.8% depending on the conditions of the study. Oil recovery from 15.0 g canola flakes loaded to the extractor was 90% after a 6 h run at a CO_2 flow rate of 3.9 L/min (measured at ambient conditions) at 24 MPa and 35°C. A higher recovery could have been achieved if the extraction were continued longer. There are also some losses during the depressurization step. The analysis of the crude *n*-hexane extracted oil indicated the presence of 0.81% (w/w) FFA which is consistent with the reports of Canola Council of Canada (1994). Blank extraction runs carried out with no enzyme loaded in the reactor at different conditions of this study indicated the presence of FFA in the

extracts. A typical SFC chromatogram of the reaction product mixture from the on-line hydrolysis of extracted canola oil is given in Figure 3.2. The retention times of FFA, MG, DG and TG were 10-16, 17-19, 23-27 and 27-31 min, respectively.

3.3.1. Effect of CO_2 flow rate on product composition

The effect of CO_2 flow rate on the hydrolysis of extracted canola oil in $SCCO_2$ was studied at 24 MPa and 35°C applying two different flow rates of 0.5 and 3.9 L/min, over 6 h. In the previous study (chapter 2), the flow rate effect on the hydrolysis of hexane-extracted and refined commercial canola oil was studied. In this experiment, the hydrolysis of oil extracted from a limited quantity of canola flakes is investigated. In this case, a change in the CO_2 flow rate results in a change in the quantity and composition of the extracted oil as a function of time in addition to a change in the residence time in the reactor.

3.3.1.1. Extraction of canola oil at different CO_2 flow rates

Figure 3.3 presents the extraction curves obtained at two CO_2 flow rates over 6 h with and without the presence of enzyme in the reactor. At the lower flow rate, the extraction curve was linear throughout 6 h and thus there was no change in the supply of oil to the enzyme bed throughout the entire run although some changes in the extract composition was expected. However, at the higher CO_2 flow rate (Fig. 3.3) the linearity of the extraction curve and, therefore, the consistency of oil supply to the enzyme sites was maintained for only ~2 h. Furthermore, a persistent change in the composition of the oil supplied to the reactor adds to the complexity of the system.

Throughout the extraction at 3.9 L/min, all three phases of the extraction curve (solubility-controlled phase, transition phase and diffusion-controlled phase) are

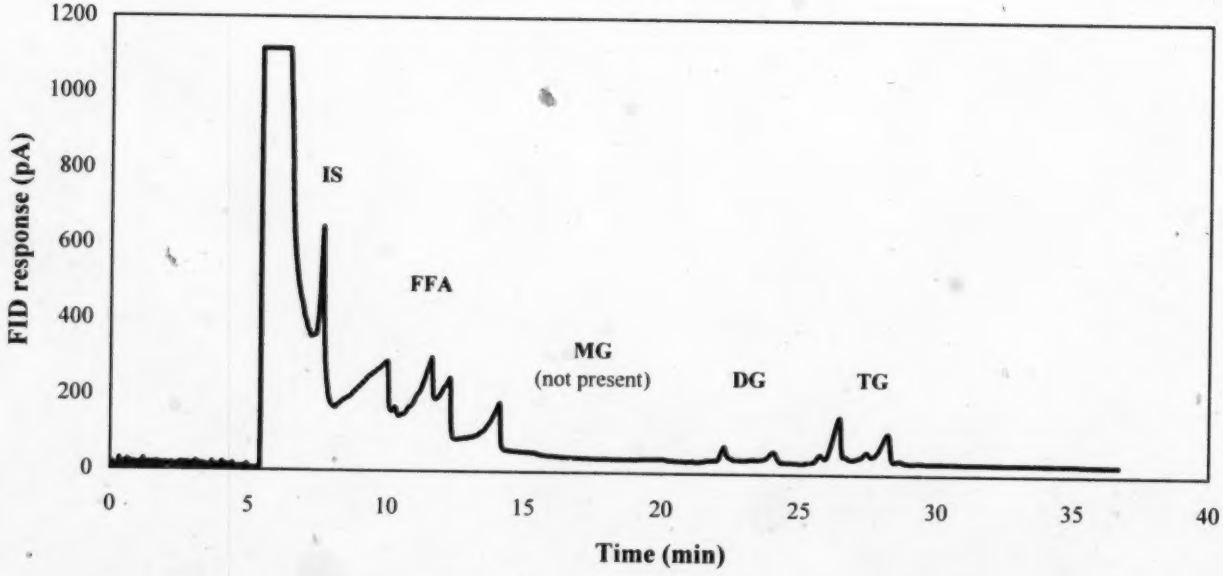

Figure 3.2. Typical supercritical fluid chromatogram of a product mixture from the on-line extraction and enzymatic hydrolysis of canola oil in $SCCO_2$; IS, internal standard; FFA, free fatty acids, MG, monoglycerides; DG, diglycerides; TG, triglycerides.

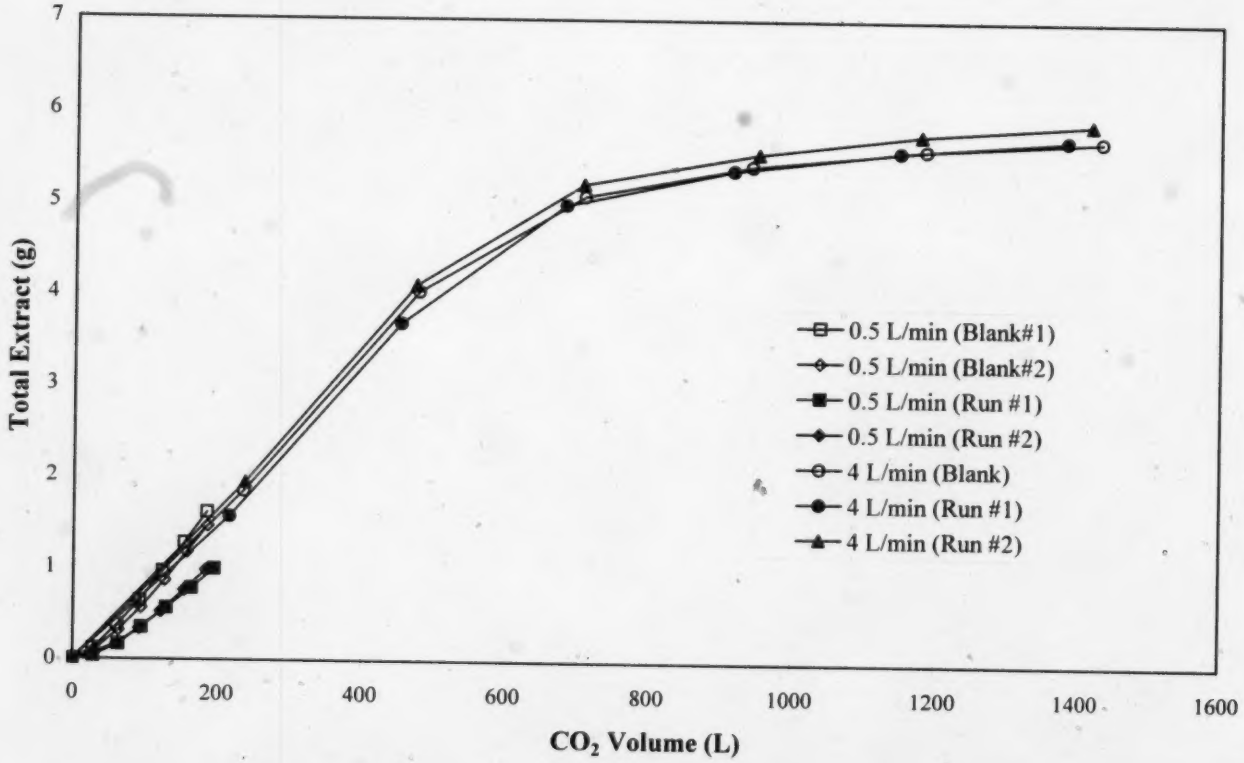

Figure 3.3. Effect of CO_2 flow rate on the total extract in the on-line hydrolysis of extracted oil from canola flakes (15.0 g) in $SCCO_2$ at 24 MPa and 35°C (enzyme load: 1.0 g, blank runs are with no enzyme; water: 0.004 mL/min).

observed (Fig. 3.3). However, extraction at 0.5 L/min exhibits only the first phase. The linear parts of the curves (i.e. solubility-controlled phase) obtained for 0.5 L/min and 3.9 L/min CO_2 flow with no enzyme present are overlapping, which indicates saturation of CO_2 with oil. However, when enzyme beads are loaded to the reactor, there was a drop in the total extract obtained at 0.5 L/min CO_2 flow, but there was no change in the extraction curve obtained at higher CO_2 flow rate (Fig. 3.3). Additional resistance to flow and retention of extract by the enzyme bed was significant at low CO_2 flow rate.

The composition change in the extracts of blank runs at 0.5 and 3.9 L/min throughout the extraction are shown in Figures 3.4 and 3.5, respectively. When CO_2 flow rate is 0.5 L/min, FFA are extracted at a higher rate than TG at the beginning of the run, which slowly declines throughout the 6 h period. This is due to the higher volatility of FFA compared to TG. In addition, CO_2 preferentially solubilizes the lower molecular weight FFA first. Selectivity shifts to TG as FFA are depleted with time. Thus, the profile of the extracted oil is changing from one high in FFA at the beginning to one high in TG towards the end of the run at a CO_2 flow rate of 0.5 L/min. Such a pattern is not observed at 3.9 L/min CO_2 flow rate. However, this effect is hidden in the first hour of the run at the higher flow rate since a large amount of extract (~2 g) is obtained (Fig. 3.3) and the FFA are diluted. In this case, an increase in the FFA content is observed after 4 h (Fig. 3.5) as oil in the flakes is depleted and the amount of extract collected in the last three fractions is quite small (Fig. 3.3). It is also possible that some FFA is produced in the absence of enzyme as a result of the presence of water and CO_2 at high pressure.

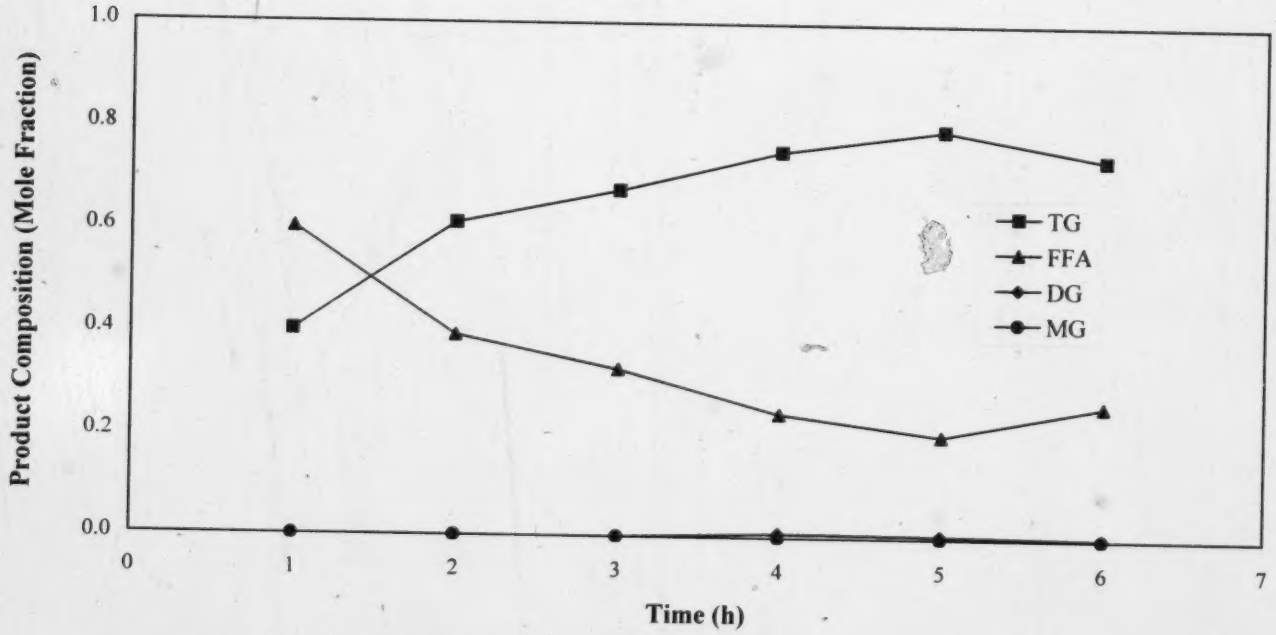

Figure 3.4. Composition of oil extracted from canola flakes (15.0 g) using $SCCO_2$ at 24 MPa and 35°C (CO_2 flow rate: 0.5 L/min, measured at ambient conditions; water: 0.004 mL/min; no enzyme).

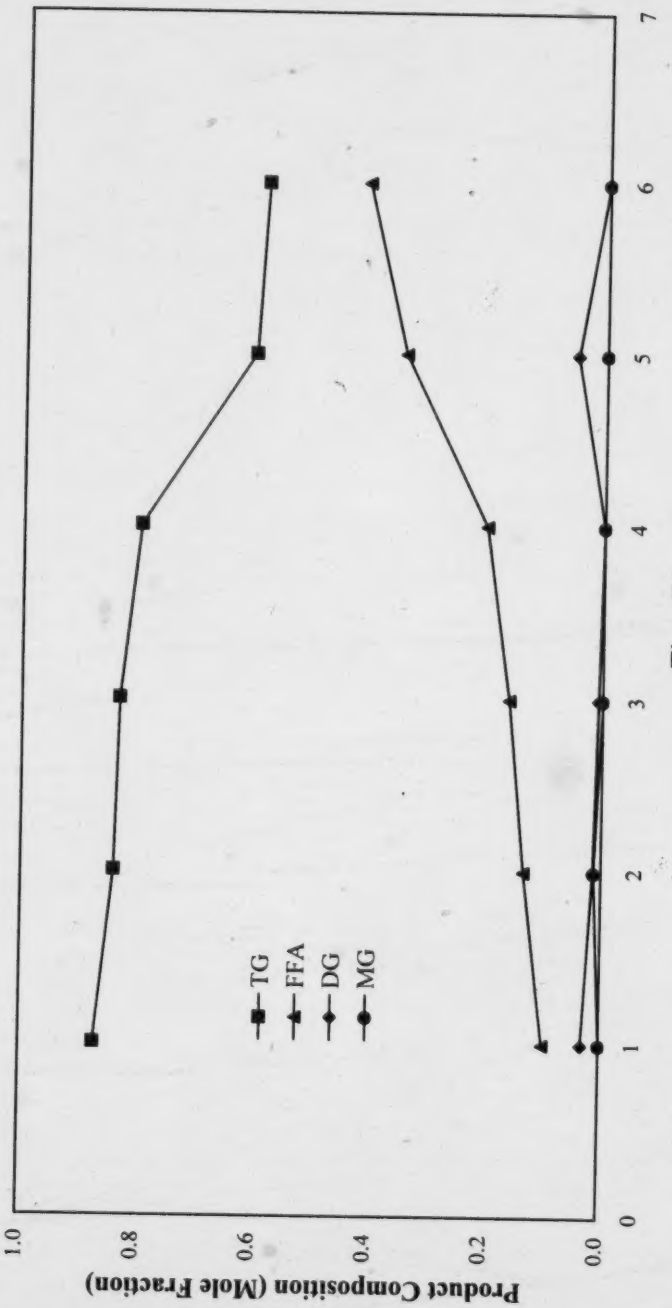

Figure 3.5. Composition of oil extracted from canola flakes (15.0 g) using $SCCO_2$ at 24 MPa and 35°C (CO_2 flow rate: 3.9 L/min, measured at ambient conditions, water flow rate: 0.004 mL/min, no enzyme).

3.3.1.2. Extraction-reaction of canola oil at different $SCCO_2$ flow rates

As shown in Figure 3.4, extracted oil composition changed substantially at a CO_2 flow rate of 0.5 L/min. It is important to know the extract composition to use as a base for the determination of enzyme performance in the hydrolysis of canola oil. FFA content of the extract fraction collected in the first hour was very high, ~60% (mole %). The main source of this FFA is from the canola flakes, which are preferentially extracted compared to TG at the initial stage of extraction. A comparison of the composition of products obtained from extraction-reaction runs carried out with and without the presence of enzyme would be a good indication of the FFA produced by the enzyme. Such point to point comparison of product composition is presented in Figure 3.6 for an enzymatic run at 0.5 L/min CO_2 flow rate. TG and FFA contents of the product of the enzymatic reaction are fairly constant throughout the entire run. There was a substantial drop in TG content with a corresponding increase in FFA content due to enzymatic hydrolysis.

Analysis of the extract fractions (i.e., no enzyme present) obtained at 3.9 L/min CO_2 flow rate indicated a steady increase in the FFA content throughout the 6 h run (Fig. 3.5). A substantial increase in the FFA content was observed after 4 h, where extraction kinetics was taken over by the diffusion of oil from the interior of flake particles into the bulk CO_2 due to depletion of freely available oil. However, when the enzyme is present to catalyze the reaction, there was a substantial drop in TG and an increase in FFA content of products (Fig. 3.7). It is important to note that the difference in the FFA content of products obtained in the blank and enzymatic runs after the 2^{nd} h is almost constant (Fig. 3.7) indicating a consistent production of FFA by the enzyme.

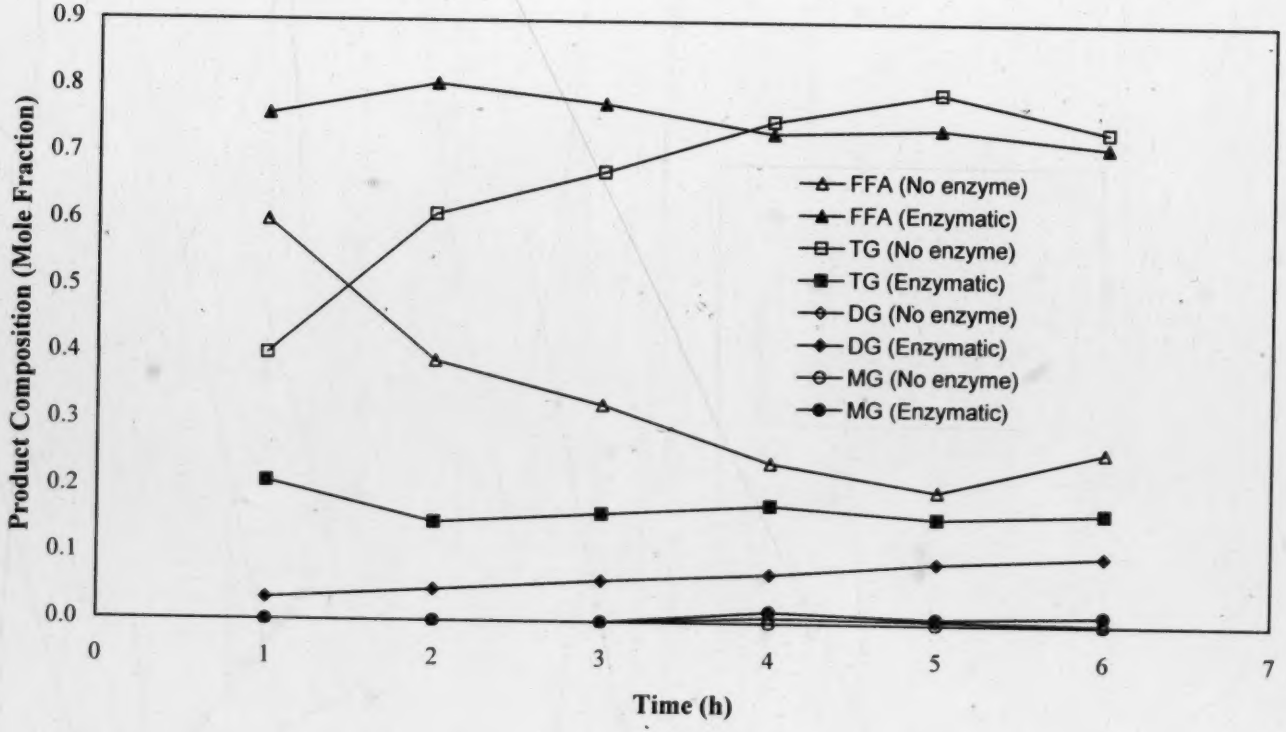

Figure 3.6. Change in product composition during on-line hydrolysis of extracted oil from15.0 g canola flakes at 24 MPa and 35°C and 0.5 L/min CO_2 flow rate, measured at ambient conditions (enzyme load: 1.0 g, water flow rate: 0.004 mL/min).

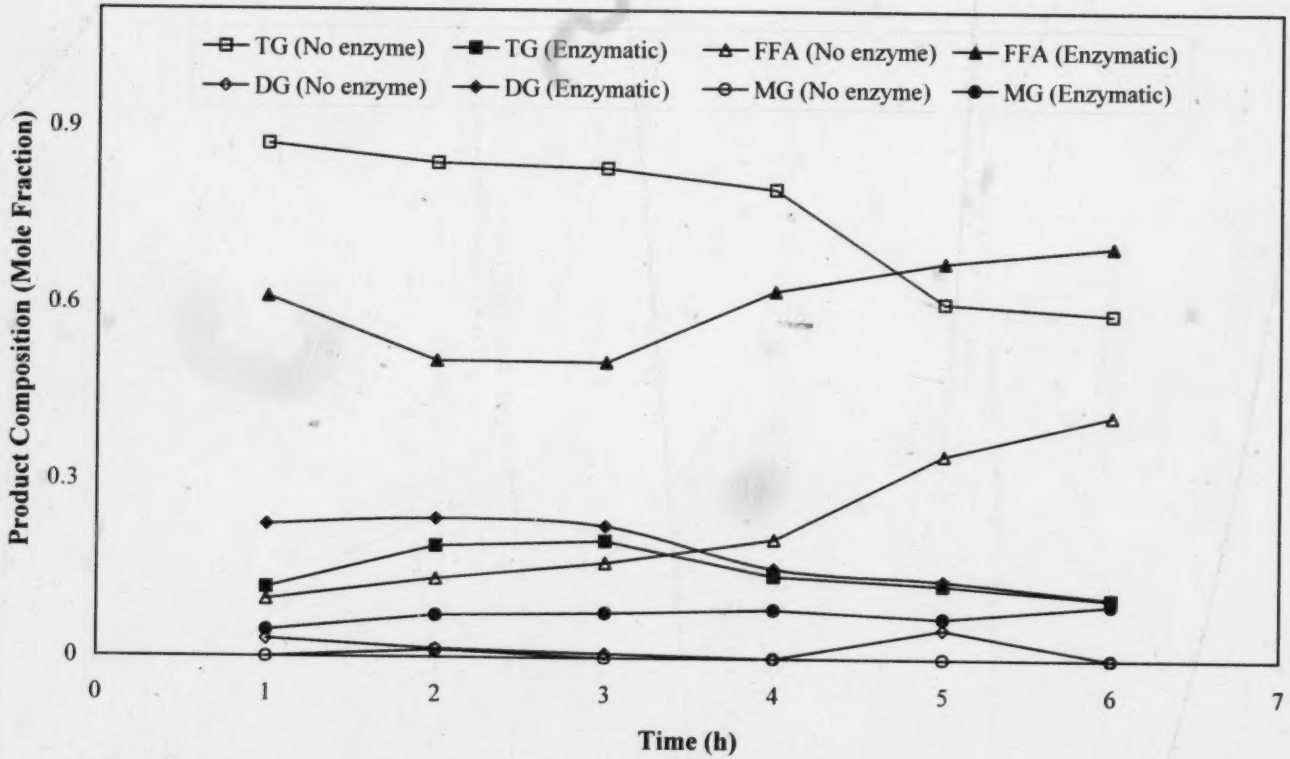

Figure 3.7. Change in product composition during on-line hydrolysis of extracted oil from 15.0 g canola flakes in $SCCO_2$ at 24 MPa and 35°C (CO_2 flow rate: 3.9 L/min, measured at ambient conditions; water flow rate: 0.004 mL/min; enzyme: 1.0 g).

Figure 3.8 compares the product composition for the two different CO_2 flow rates. MG and DG contents of the products obtained at the higher flow rate were higher than those at the lower flow rate. This is mainly due to the low residence time for the excessive amount of extracted TG to contact the enzyme. Therefore, at the higher CO_2 flow rate partial hydrolysis dominates the reaction during the first 3 h. However, after the third hour as a result of a drop in the amount of extract, MG and DG are further hydrolyzed. This is evident by a drop in their level and a corresponding increase in the FFA production.

In the case of the reaction conducted at the lower CO_2 flow rate, the MG and DG contents increased with time. This is partly due to a gradual increase in the supply of TG and a corresponding decrease in the supply of FFA to the reactor (Fig. 3.4) at the lower CO_2 flow rate. In addition, the overall quantity of TG supplied to the enzyme bed is much less than that at the higher flow rate (Fig. 3.3). Furthermore, the residence time in the reactor is longer at the lower flow rate. Therefore, the enzyme has better chance to almost fully hydrolyze the TG to glycerol and as a result, the formation of the intermediate products (MG and DG) is not favored.

3.3.2. Effect of enzyme load on the total extract and composition of the product mixture

The effect of enzyme load at 1.0, 2.0 and 5.0 g levels on the FFA production is shown in Figure 3.9 for an extraction-reaction at 24 MPa and 35°C. The FFA production during blank runs carried out with no enzyme is also shown for comparison. Since FFA are more soluble in $SCCO_2$ than TG, an earlier extraction of FFA is expected, which is evident in the product composition curves given in Figure

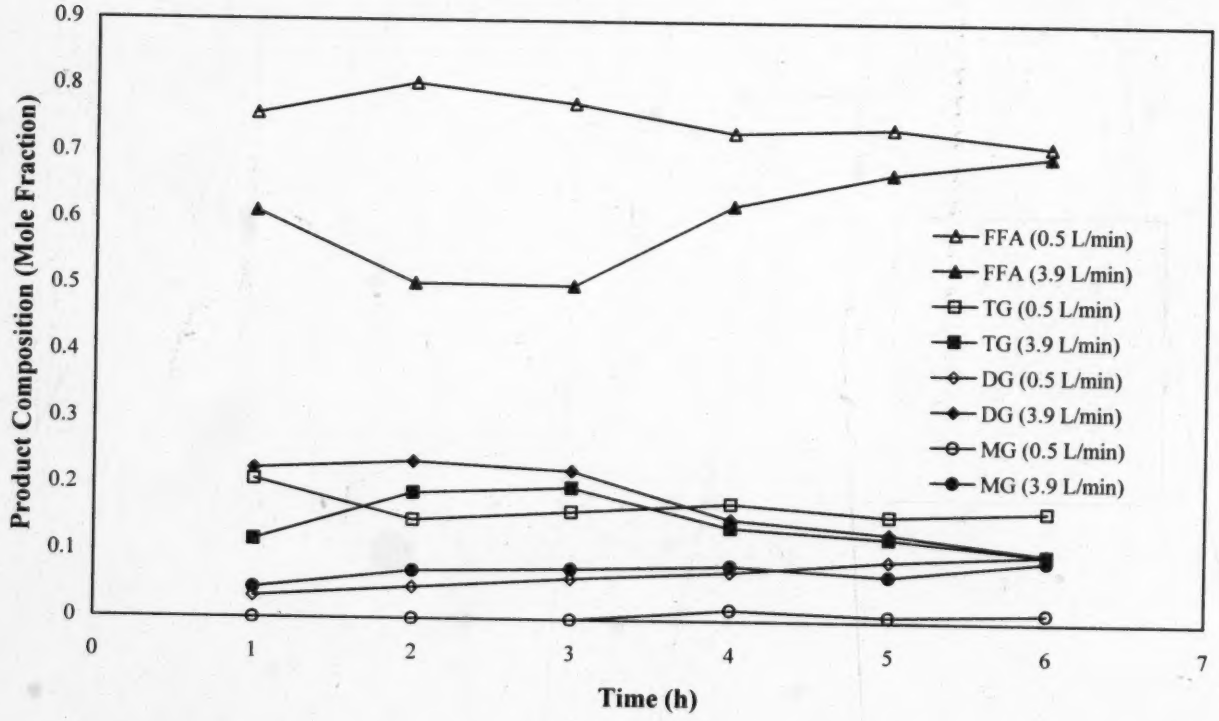

Figure 3.8. Effect of CO_2 flow rate, measured at ambient conditions, on the composition of the product mixture in the on-line hydrolysis of extracted oil from canola flakes in $SCCO_2$ at 24 MPa and 35°C (Water flow rate: 0.004 mL/min, enzyme: 1.0 g).

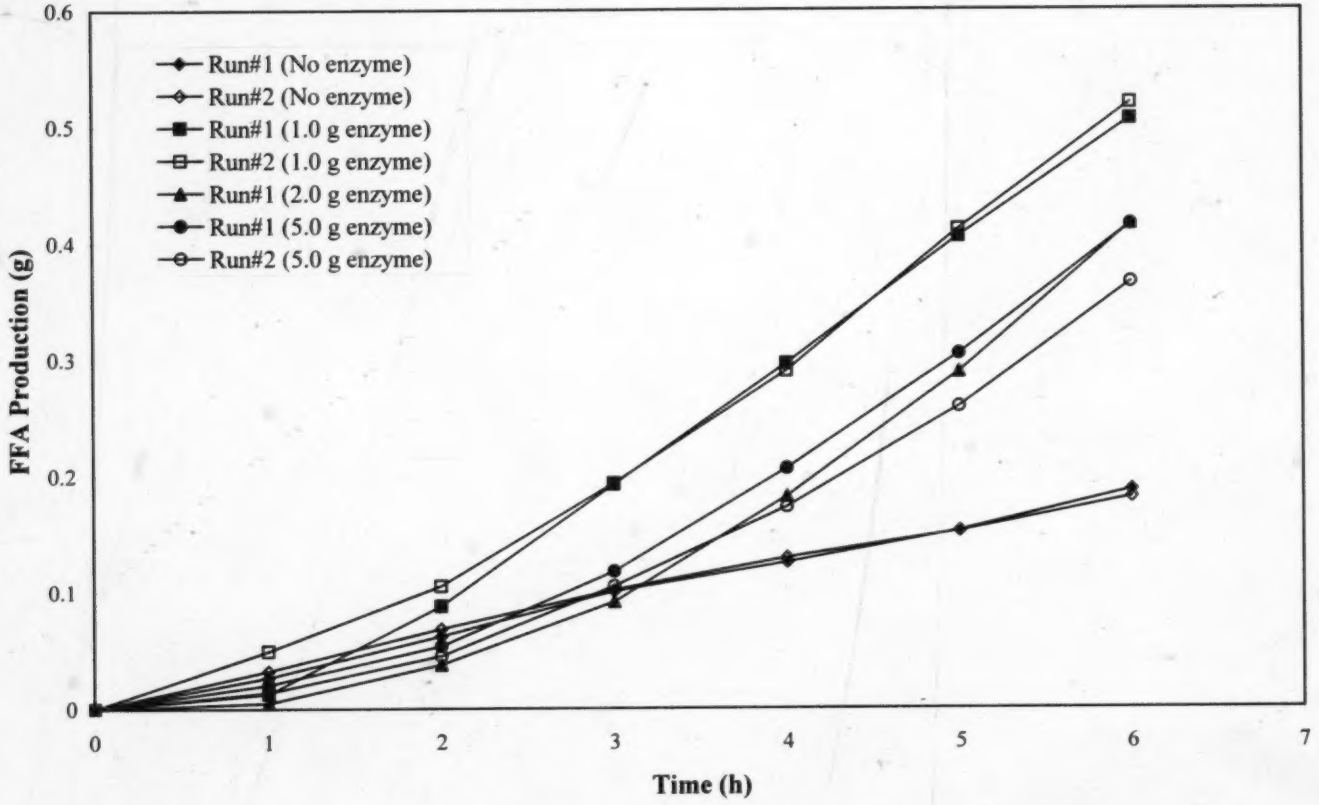

Figure 3.9. Effect of enzyme load on the FFA production in the on-line hydrolysis of oil from 15.0 g canola flakes in $SCCO_2$ at 24 MPa and 35°C (CO_2 flow rate: 0.5 L/min, measured at ambient conditions, water flow rate: 0.004mL/min).

3.4. In addition, some FFA formation may occur due to supercritical conditions. A positive deviation in the curves of enzyme-catalyzed reactions from those of the blank indicated the extent of FFA production by the enzyme (Fig. 3.9). The largest level of FFA production was achieved with 1.0 g enzyme. A higher level of FFA production was anticipated when a higher level of enzyme load was used. On the contrary, less FFA was produced when 2.0 g or 5.0 g of enzyme was loaded into the reactor. The nature of the packed bed of immobilized enzyme and glass beads may be responsible for this result since a tightly packed reactor introduces more resistance to the flow of oil-laden $SCCO_2$. In fact, when the amount of total extract obtained with different reactor packing was compared (Fig. 3.10), a substantial drop due to the increased bed resistance was observed at a CO_2 flow rate of 0.5 L/min. The highest extraction level was obtained when there was no enzyme present in the reaction cell; whereas, the enzyme loads of 2.0 and 5.0 g resulted in the lowest amount of extract.

The different amounts of glass beads used to disperse the immobilized enzyme beads would affect the bed packing and the flow behavior through the reactor. The proportion of the glass beads to the enzyme was changed based on the limited reactor volume, where 15.8 and 2.7 g glass beads were used for 2.0 and 5.0 g enzyme loads, respectively. When a lower level of oil is introduced onto a larger amount of enzyme, oil will have more opportunity to contact the many layers of enzyme in the reaction cell. This is verified by a higher mole fraction of the FFA as well as a lower level of the TG in the run with 5.0 g enzyme, where a lower DG and a higher MG levels are observed (Fig. 3.11). Therefore, complete hydrolysis of oil is more pronounced with a higher level of enzyme in the system. However, the extent of improvement in the degree of hydrolysis is not proportional to the increase in the enzyme load.

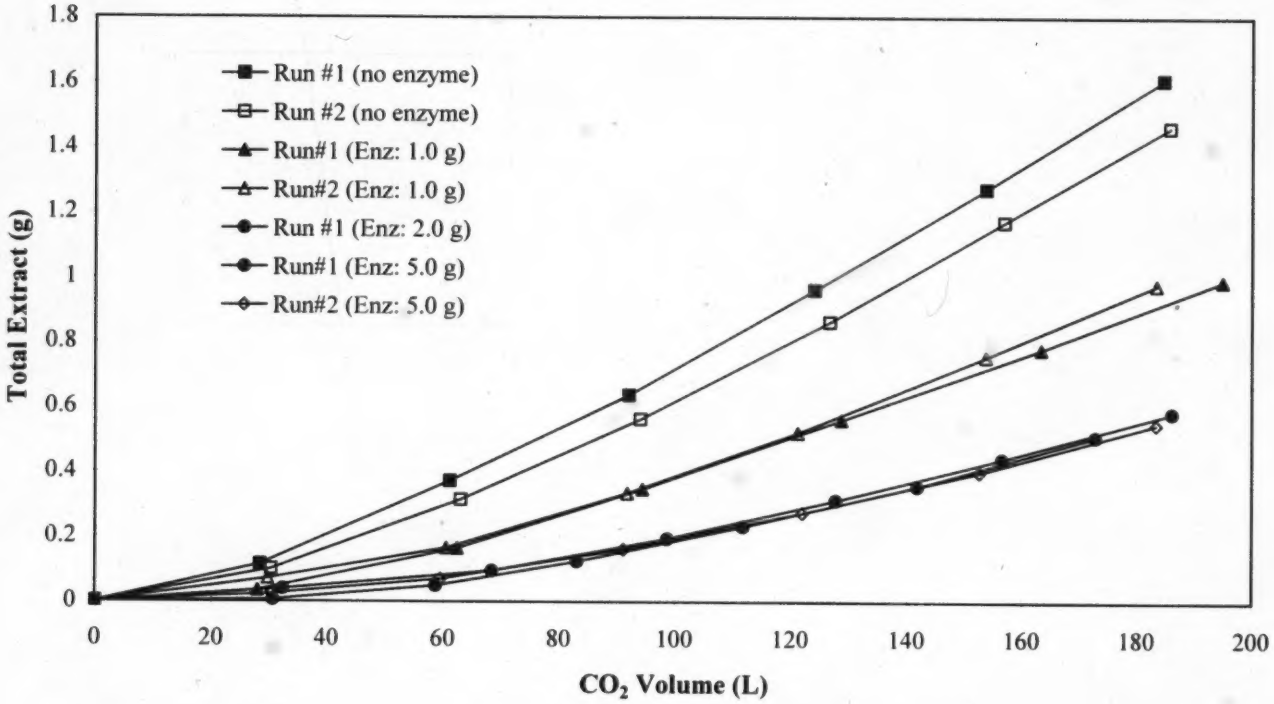

Figure 3.10. Effect of enzyme load on the total extract in the on-line hydrolysis of oil from 15.0 g canola flakes in $SCCO_2$ at 24 MPa and 35°C (CO_2 flow rate: 0.5 L/min, measured at ambient conditions; water flow rate: 0.004 mL/min).

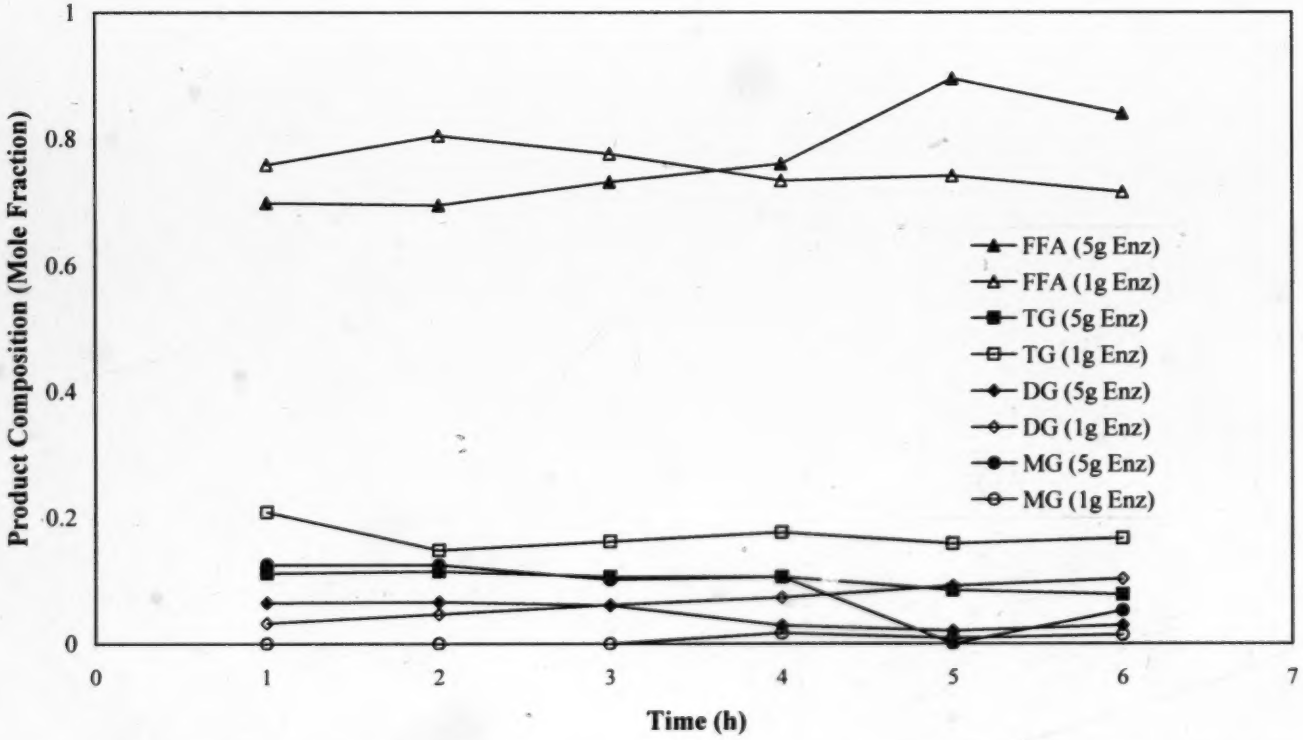

Figure 3.11. Effect of enzyme load on the product composition in the on-line hydrolysis of oil from 15.0 g canola flakes in $SCCO_2$ at 24 MPa and 35°C (CO_2 flow rate: 0.5 L/min, measured at ambient conditions; water flow rate: 0.004 mL/min).

The enzyme performance quantified by the amount of FFA produced in the reaction did not improve with an increase in the amount of enzyme in the reaction chamber (Fig. 3.9). This is related to a reduction in the total amount of extract (Fig. 3.10), which could be attributed to the scale-up in the enzyme load and the retention of some of the extract by the enzyme bed. The increase in the enzyme load was not compensated for by a proportional increase in the geometrical dimensions of the reaction chamber. Hampson and Foglia (1999) reported that a higher enzyme bed volume was crucial for the solubilized substrates to have better contact with the enzyme. In addition, extra water may be needed to make up for the water consumption due to increased enzyme load, but it was not compensated for in this study. Hampson and Foglia (1999) reported that the immobilized lipase from *C. antarctica* lost 2-6% (w/w) of its water content per hour in $SCCO_2$ at 27 MPa and 60°C with 0.5 or 1 L/min CO_2 flow rate. They also reported that in the hydrolysis of tripalmitin, the enzyme with 1.5% (w/w) moisture content gave little evidence of hydrolysis. However, the enzyme with 5.4-23.5% (w/w) initial moisture content led to products with palmitic acid and unreacted tripalmitin only, which is in agreement with the results of the lower flow rate in this study. A lack of water may be a source of problem and encourages more research to better understand the effects of water.

3.3.3. Effect of canola load on product composition

The effect of the quantity of the cooked canola flakes used for extraction on the reaction product composition is shown in Figure 3.12 for two different sizes of canola load (3.0 and 15.0 g). A higher FFA and a lower DG content associated with a smaller size of canola load indicated the extent of hydrolysis of TG to be greater. The

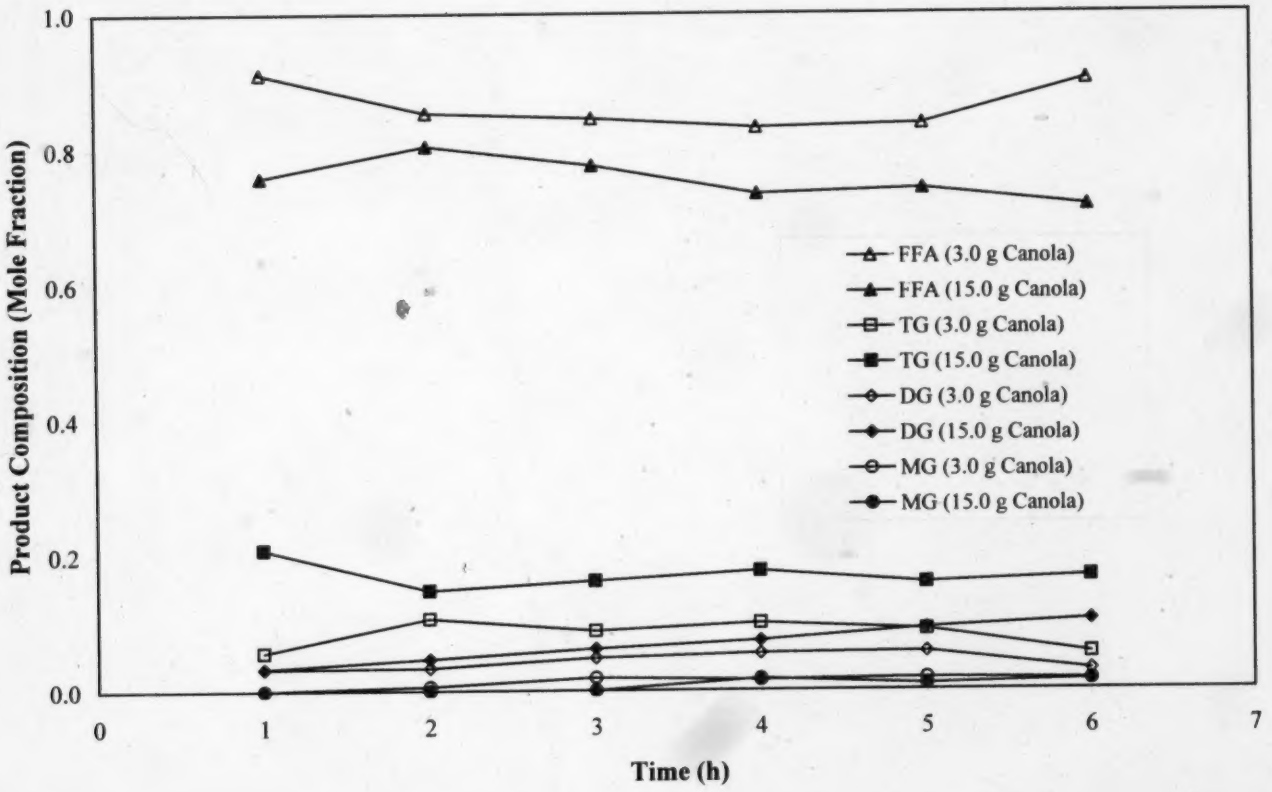

Figure 3.12. Effect of canola load on product composition in the hydrolysis of oil from canola flakes in $SCCO_2$ at 24 MPa and 35°C (CO_2 flow rate: 0.5 L/min, measured at ambient conditions; enzyme: 1.0 g; water flow rate: 0.004 mL/min).

distinction between the DG curves for the two conditions is becoming more pronounced with time since the supply of oil is much more limited with a 3.0 g batch compared to that of 15.0 g canola flakes. Such depletion was evident in a curvature, which was not yet in the diffusion-controlled area of the extraction curve for the 3.0 g canola load after 4 h. A higher concentration of TG in the 15.0 g batch is another reason for the lower conversion rate in this case. The MG concentration was very low for both cases. However, with the 15.0 g batch, MG appeared in the extract at a later time as a result of a drop in the level of other components.

It is possible to somewhat improve the concentration of one species such as DG in this case by controlling the amount of canola flakes in the extraction chamber. Adjusting this parameter along with CO_2 flow rate and enzyme load can improve the concentration of species of interest especially if they are combined with pressure and/or temperature manipulation.

3.4. CONCLUSIONS

Enzyme load, CO_2 flow rate and quantity of canola flakes are parameters that influence the on-line lipase-catalyzed hydrolysis of the extracted oil from cooked canola flakes. An on-line extraction-reaction system gives an opportunity to study the reaction profile where a steady change in the feed oil load and its composition in the $SCCO_2$ exists. An increase in the oil load resulted in reduced FFA production at the earlier stages of the run. However, towards the end of the run where the supply of oil to the enzyme was limited, FFA concentration in the product improved. By increasing the load of canola flakes, a clear improvement in the concentration of DG in the product samples was observed.

To take full advantage of the processing improvements developed in this study without compromising the production rate of the reaction, certain scale-up parameters such as a change in the dimensions of the extraction and reaction chambers have to be considered. For optimum and consistent reactor performance, extractor load needs to be replaced when the extraction curve enters the diffusion-controlled region. Extraction and reaction conditions need to be optimized to maximize the yield of the species of interest.

On-line extraction-reaction of lipids shows potential for process development to produce various basic ingredients like FFA, glycerol, MG, DG, other nutritionally important TG such as those high in ω-3 fatty acids, and fatty acid esters for industries such as foods and drugs, soap and detergents, surfactants and other health and hygiene related products.

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4. MICROSTRUCTURAL ANALYSIS OF LIPASE USED IN THE HYDROLYSIS OF CANOLA OIL IN $SCCO_2$[1]

4.1. INTRODUCTION

Studies have shown that free enzymes; i.e. not immobilized, have different sizes, polarizability, chemical structure and protein content and therefore have different properties in terms of their specificity and selectivity in catalyzing different reactions. In cases where enzymes in their non-immobilized states are used, they are dissolved in a solvent such as water or an organic solvent. However, when they are immobilized, they are bound to a solid material such as glass beads (Miller et al., 1991) and natural or synthetic materials including bioskins (Legaz et al., 1998), silk fibroin membrane (Liu et al., 1995) and polyaniline films (Verghese et al., 1998). Attachment of enzyme to the solid support is facilitated by different ways including ionic attractions and covalent bonds. Depending on the method of immobilization and type of enzyme, they may loose part of their activity or become more stable after immobilization.

Enzyme inactivation is not necessarily due to some physical structural changes. Kamat et al. (1995) using Laser Desorption Mass Spectrometry (LD/MS) reported that CO_2 under supercritical environment formed a covalent bond with the lipase and inhibited its activity. Scanning Electron Microscopy (SEM) is a technique that may be used to view microstructural changes outside the surfaces. Vasudevan and Weiland (1994) reported that the microstructure of bovine liver catalase and

[1] A version of this chapter is to be submitted to the Journal of Agricultural and Food Chemistry for consideration for publication.

Aspergillus catalase did not undergo any changes with a chemical deactivation method using hydrogen peroxide.

Since enzymes vary in size, their structure may appear differently if they are observed under an electron microscope. Smaller enzymes can stay very close to each other during immobilization and therefore a higher magnification will be required. On the other hand, larger enzymes can be visible with a lower magnification. For example, while the enzyme *Aspergillus* catalase was hardly visible under an electron microscope at 7,000 magnification, bovine liver catalase was very easily observed at 560X magnification (Vasudevan and Weiland, 1994).

Although various studies investigated the effect of pressure and other parameters such as temperature, water content and type of the supercritical fluid on enzyme activity, our understanding of structural changes that may occur when an enzyme is exposed to high pressure in supercritical media is quite limited.

Numerous studies have reported a loss in enzyme activity and/or stability after exposure to certain conditions such as high pressure (Kamat et al., 1992; Yu et al., 1992; Lee et al., 1993) or excessive water content (Marty et al., 1992). The extent of inactivation had a direct negative effect on the reaction process in terms of reaction rate and the amount of products etc. On the other hand, there are reports claiming an increase in enzyme activity with increasing pressure (Ikushima et al., 1995). It is well known that enzymes exposed to ultra high pressures (>400 MPa) undergo an irreversible structural change, which will result in complete inactivation (Randolph et al., 1991). However, the literature lacks information on the structural changes of immobilized enzymes exposed to range of conditions typical in supercritical studies (≤40 MPa). Therefore, the objective of this study was to examine any changes in the

structure of immobilized lipase from *Mucor miehei*, Lipozyme IM, under supercritical conditions using SEM. The effects of pressure, CO_2 flow rate, enzyme load and oil content were investigated.

4.2. MATERIALS AND METHODS

4.2.1. Materials

Cooked canola, *Brassica napus* and *B. campestris*, flakes and *Mucor miehei* lipase immobilized on macroporous anionic resin, Lipozyme IM, were kindly provided by Canamera Foods (Fort Saskatchewan, AB) and Novo Nordisk (Franklinton, NC), respectively. Enzyme was stored at 5°C until used in the experiments. CO_2 used as reaction medium, bone dry (99.8%) was purchased from Praxair (Mississauga, ON).

4.2.2. Experimental setup and design

The on-line extraction-reaction of oil from canola flakes was performed using the system described in section 3.2.2. Experiments were conducted at two different pressures (24 and 38 MPa) and 35°C. CO_2 flow rate (0.5 and 3.9 L/min, measured at ambient conditions), level of enzyme load (1.0 and 5.0 g) and amount of canola flakes (3.0 and 15.0 g) used as the source of canola oil were the parameters studied. Reactions were continued for 6 h and then enzyme batches were taken out of the reactor after a slow depressurization (~30 min) and analyzed by SEM.

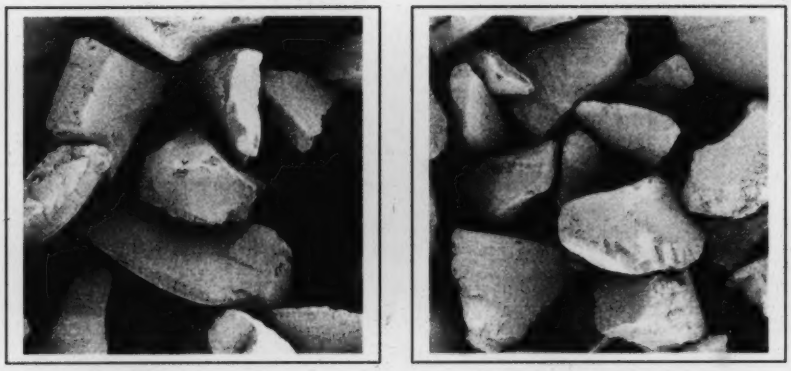
4.2.3. Scanning Electron Microscopy

A JEOL 6301 FXV scanning electron microscope (Peabody, MA) was used for the analysis of the immobilized lipase before and after a reaction in $SCCO_2$ media. Typical representatives of different batches of enzyme used at various reaction conditions studied were selected for SEM analysis. Two different coating methods

were applied for sample preparation. In the first method, each enzyme grain was fixed on a specimen mounting stub using a double-sided conductive carbon tape and then gold-sputtered in argon atmosphere using a Nonotech semprep2 system (Neuilly, France). In the second method, enzyme grains were attached to the SEM stubs using conductive silver paint, which was allowed 5 days to dry before use. Then, the grains were sputter-coated with chromium in xenon atmosphere using an Edward high vacuum XE 200 xenosput (Crawley Sussex, England) and analyzed. The second method was used to achieve better enzyme attachment to the specimen mounting stubs so that the enzyme grains would not move during SEM analysis. In addition, a thinner layer of coating was possible with chromium and therefore a clearer image was expected. In both methods some moving was observed occasionally and results from samples with severe moving problems were not included.

4.3. RESULTS AND DISCUSSION

Figure 4.1 compares the micrographs of the immobilized enzyme beads before and after a reaction in $SCCO_2$ medium at 38 MPa and 35°C for 6 h. At the magnification level of these micrographs (55X), no apparent differences were observed. The grains in the picture (size range: 0.2-0.6 mm) belong to the macroporous anionic exchange resin used by the manufacturer for enzyme immobilization and the *Mucor miehei* lipase is strongly bound to the resin by adsorption. The resin is of the phenolic type and no crosslinking agents were used by the manufacturer. A typical image area where the SEM micrographs were prepared is shown in Figure 4.2 at 300X magnification level. While bovine liver catalase was easily visible at 560X magnification (Vasudevan and Weiland, 1994), the enzyme



(a) (b)

Figure 4.1. Micrographs of immobilized lipase from *Mucor miehei* before (a) and after (b) a treatment with $SCCO_2$ at 38 MPa and 35°C for 6 h. (CO_2 flow rate: 0.5 L/min, measured at ambient conditions). The image was reduced 15 times from an original 55X magnified graph.

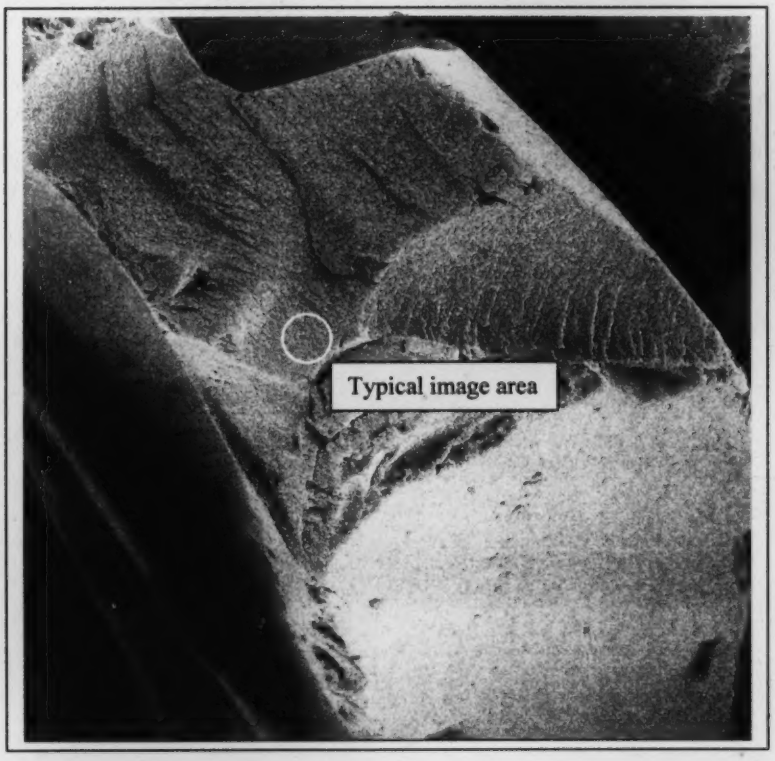


Figure 4.2. A typical image area on the grains of immobilized lipase from *Mucor miehei* where the SEM micrographs were prepared. The image was reduced 3 times from an original 300X magnified graph.

from *Mucor miehei* in this study could not be observed in 1,000X enlarged micrographs (Fig. 4.3). Therefore, all of the remaining images (Figs. 4.4-4.7) were reported at 50,000X magnification. Neither the free enzyme (i.e. not immobilized on any support) nor the enzyme-free anion exchange resin was available. However, based on the SEM images at 50,000X magnification obtained in this study, the spherical particles ranging in size from 35-55 nm on the outside surface of the solid support are interpreted to be a group of enzyme molecules.

4.3.1. Effect of high pressure

The micrographs of 1.0 g enzyme batches which had undergone a 6 h run at 24 and 38 MPa and 35°C in $SCCO_2$ were compared with that of an untreated enzyme in Figure 4.4. In these experiments, oil was extracted from 15.0 g canola flakes with CO_2 at a flow rate of 3.9 L/min and water was pumped into CO_2 at a rate of 0.004 mL/min. Results from the two different sputtering methods applied are presented. Micrographs on the left column of Figure 4.4 are from gold-sputtered samples and the ones to the right are from chromium-sputtered samples. There are no apparent differences attributable to the effect of pressure on the microstructure of the enzyme. Pressure at the levels of this study does not affect enzyme activity significantly (Miller et al., 1991). Usually a combination of several other factors such as excessive water content and overheating under high pressure promotes enzyme inactivation (Marty et al., 1992). Vasudevan and Weiland (1994) successfully inactivated bovine liver catalase using 0.01-1.0 M hydrogen peroxide. Although there was no pressure involved in their method, the SEM micrographs did not indicate any change in the structure of the enzyme after such treatment. Their results along with the results of this study indicate that the enzyme inactivation is not at a level to be observed by physical

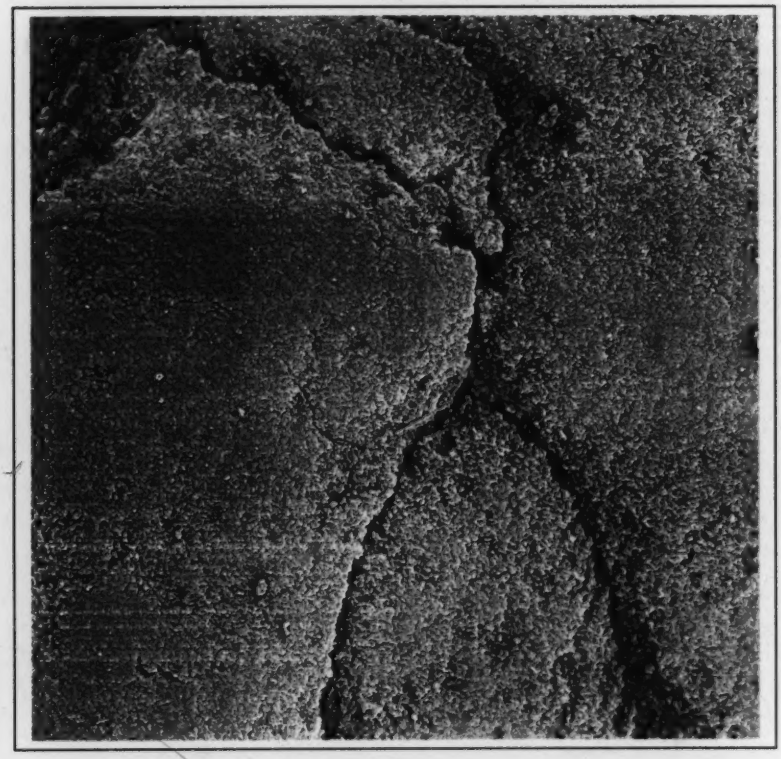


Figure 4.3. Micrograph of immobilized lipase from *Mucor miehei*. The image was reduced 3 times from an original 1000X micrograph.

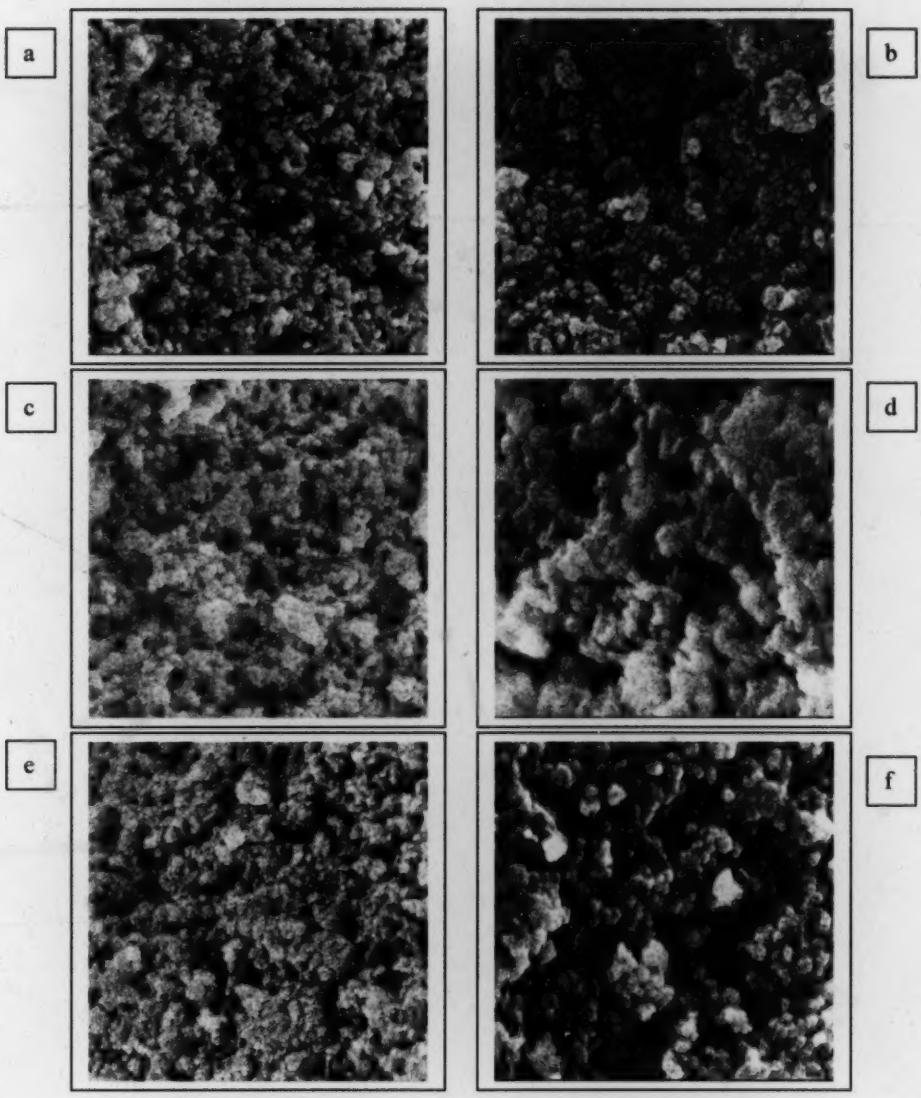


Figure 4.4. Pressure effect on the microstructure of immobilized lipase from *Mucor miehei* in the hydrolysis of canola oil in $SCCO_2$ at 35°C and 3.9 L/min CO_2 flow rate (measured at ambient conditions). a,b: untreated; c,d: 24 MPa; e,f: 38 MPa. Micrographs were reduced 15X from an original 50,000X.
Left column: samples gold-sputtered; right column: samples chromium-sputtered.

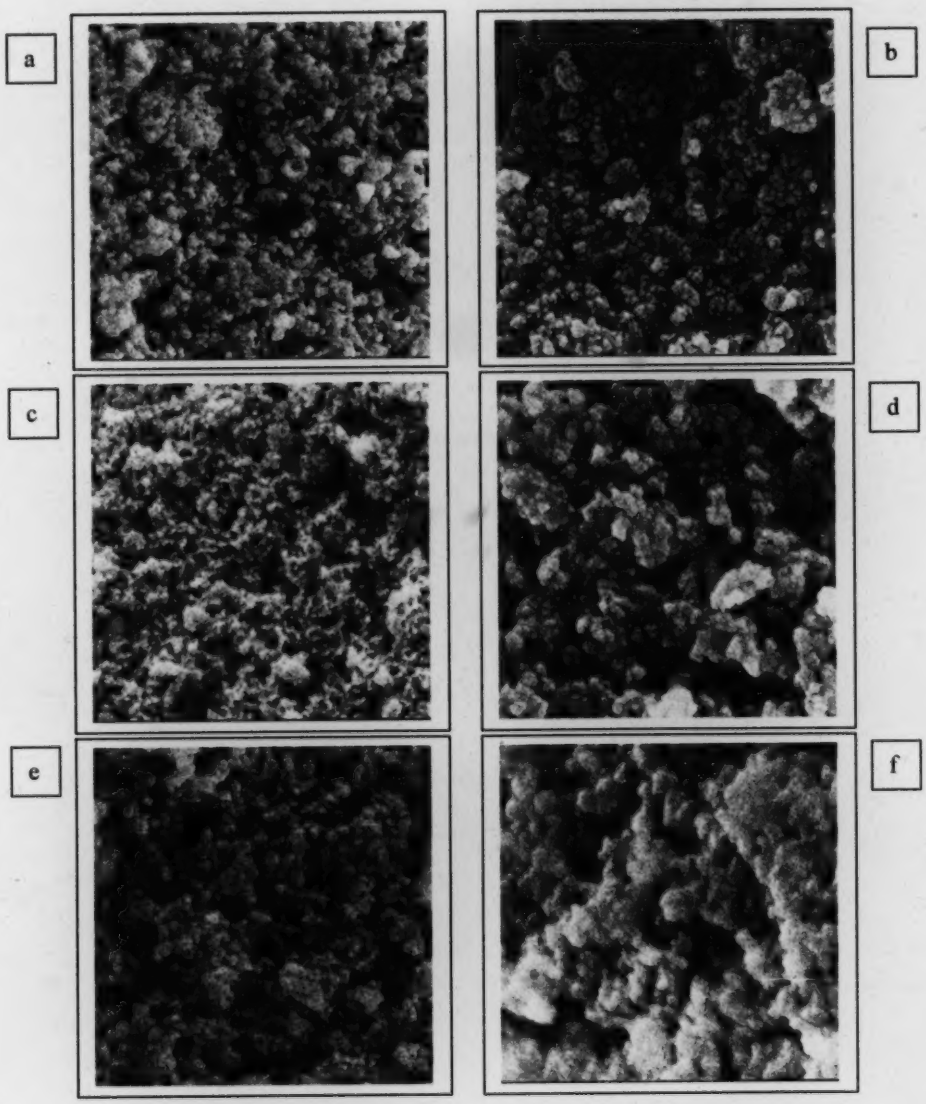


Figure 4.5. Effect of CO_2 flow rate on the microstructure of immobilized lipase from *Mucor miehei* in the hydrolysis of canola oil in $SCCO_2$ at 24 MPa and 35°C. a,b: untreated; c,d: 0.5 L/min; e,f: 3.9 L/min CO_2 flow rate, measured at ambient conditions.
Micrographs were reduced 15X from an original 50,000X.
Left column: samples gold-sputtered; right column: samples chromium-sputtered.

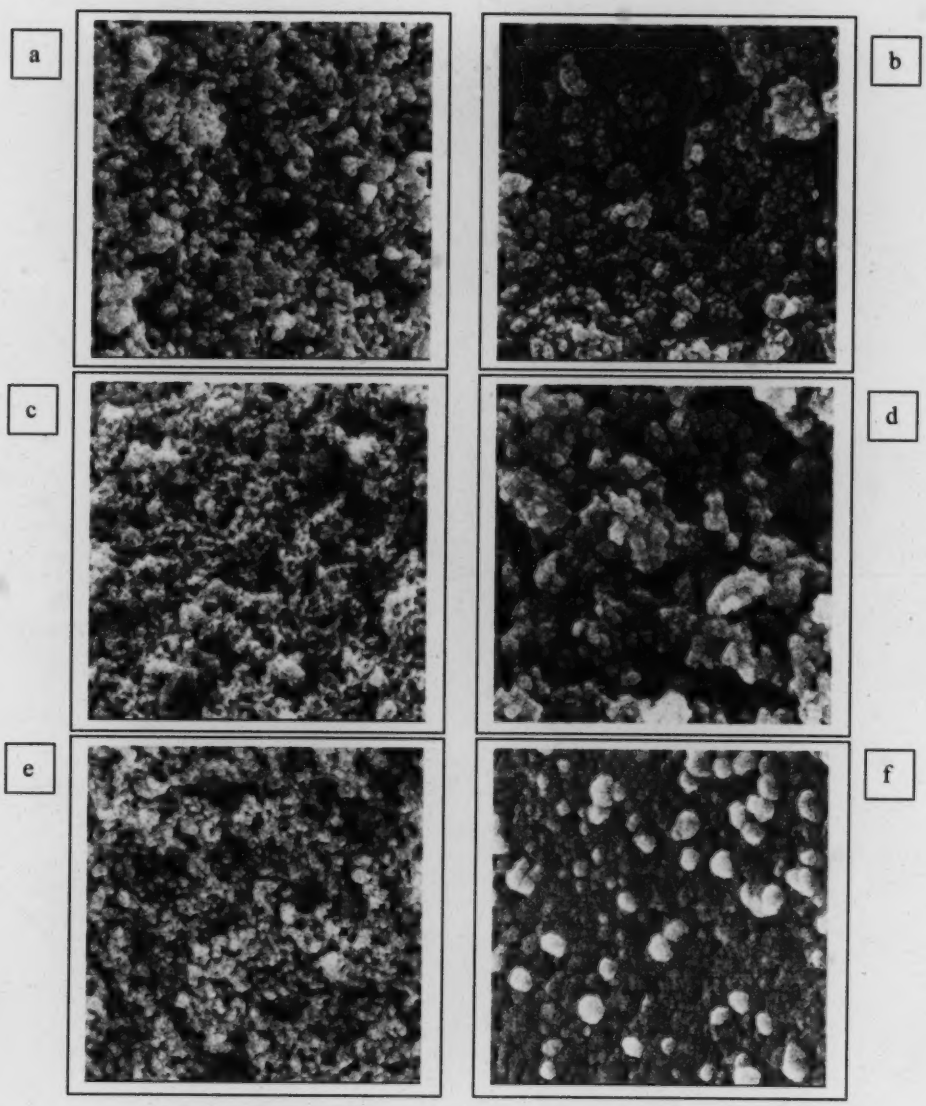


Figure 4.6. Effect of enzyme load on the microstructure of immobilized lipase from *Mucor miehei* in the hydrolysis of canola oil in $SCCO_2$ at 24 MPa and 35°C and 0.5 L/min CO_2 flow rate (measured at ambient conditions). a,b: untreated; c,d: 1.0 g enzyme; e,f: 5.0 g enzyme.
Micrographs were reduced 15X from an original 50,000X.
Left column: samples gold-sputtered; right column: samples chromium-sputtered.

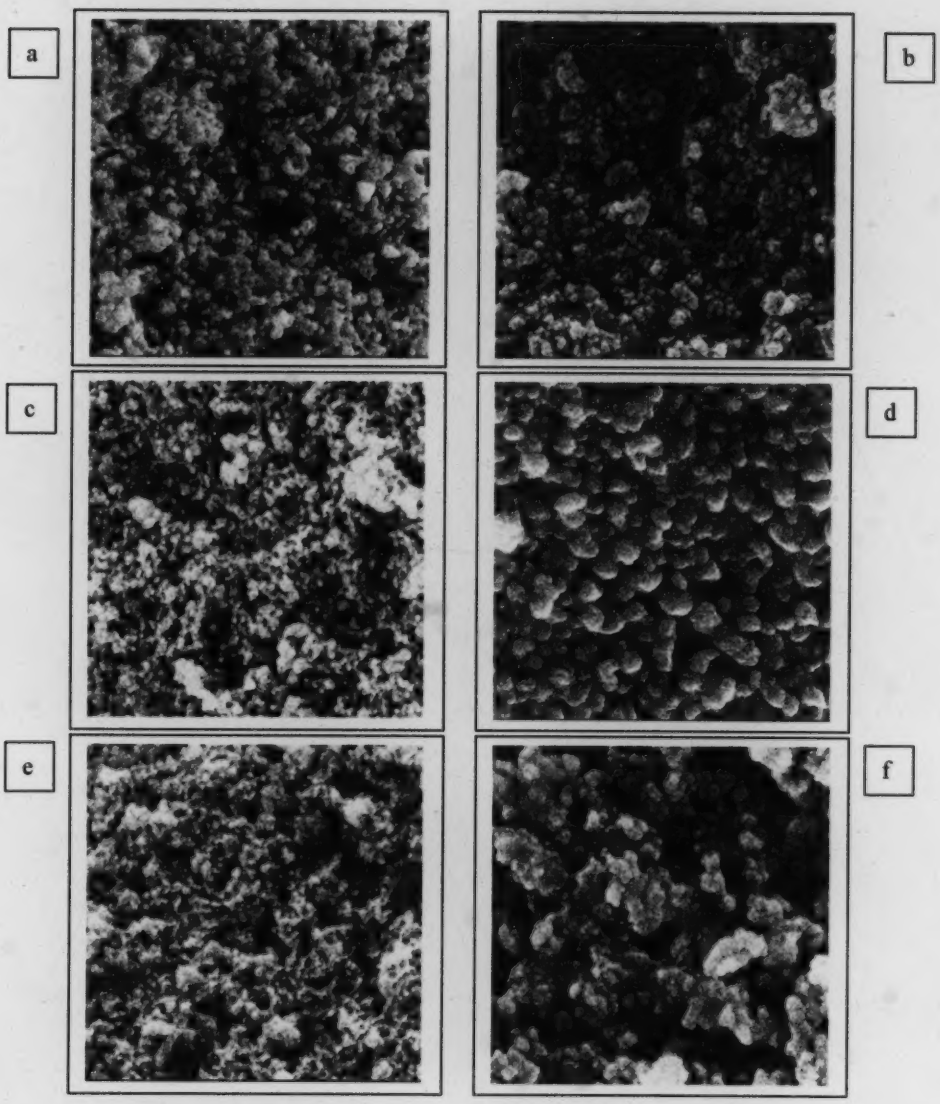


Figure 4.7. Effect of oil content on the microstructure of immobilized lipase from *Mucor miehei* in the hydrolysis of canola oil in $SCCO_2$ at 24 MPa and 35°C and 0.5 L/min CO_2 flow rate (measured at ambient conditions). a,b: untreated; c,d: oil extracted from a 3.0 g batch of canola flakes; e,f: oil extracted from a 15.0 g batch of canola flakes.
Micrographs were reduced 15X from an original 50,000X.
Left column: samples gold-sputtered; right column: samples chromium-sputtered.

structural analysis like the one performed by SEM. However, there could be some chemical deformations occurring at the inter- or intra-molecular level. If the presence of CO_2 at high pressure has had a reversible effect, it would have disappeared by the depressurization of the solvent. However, if such effects are irreversible, then physical observation by SEM may be helpful depending on the level of structural changes.

4.3.2. Effect of CO_2 flow rate

Two separate enzyme batches (1.0 g each) were exposed to $SCCO_2$ at 24 MPa and 35°C at two different CO_2 flow rates of 0.5 and 3.9 L/min, measured at ambient conditions, over 6 h. The micrographs of the treated enzymes were compared with that of untreated enzyme (Fig. 4.5). There were no apparent changes in the physical structure of the enzyme before and after exposure to CO_2 flow at these levels. Therefore, either it is not possible to identify any changes by SEM at this level of magnification (50,000X) or there are no structural changes due to these conditions.

4.3.3. Effect of enzyme load

Structural changes that might result from different enzyme loads were investigated using a 1.0 and a 5.0 g enzyme batch in $SCCO_2$ at 24 MPa and 35°C. For each run, a batch of 15.0 g canola flakes was loaded in the extraction chamber and a 0.5 L/min CO_2 flow rate was used. The micrographs were presented in Figure 4.6 in comparison to those for untreated enzyme. There were no apparent differences among the different batches of enzyme if mounted by carbon tapes and sputter-coated with gold (Fig. 4.6 a,c,e). However, when the samples were mounted using silver paint and chromium-sputtered (Fig. 4.6 b,d,f), some grains from the 5.0 g batch showed a significant difference compared to the gold-sputtered sample (Fig. 4.6 e,f). Such difference in the appearance of the enzyme bead structure is most likely a result of a

close contact among different enzyme grains leading to oil adsorption on the surface, which could not be carried away by CO_2 flow. Such oil contamination was apparent in the samples when they were removed from the reactor. In the case of 5.0 g enzyme, the ratio of glass beads to enzyme was much less than that of the 1.0 g batch (2.7:5.0 vs. 7.9:1, respectively). Such modification was necessary due to the limited volume of reactor cell. Also, a lack of sufficient water on the enzyme bed for the 5.0 g batch may have been a factor since no additional water was incorporated to compensate for the extra water requirement of this batch. Regardless of the cause(s) of such differences, the amounts of total oil extracted and total FFA produced with this batch were less than those of the 1.0 g batch (Fig. 3.10 and 3.11, respectively).

4.3.4. Effect of canola load

The effect of oil content on enzyme structure in the lipase-catalyzed hydrolysis of canola oil was investigated using two different levels of cooked canola flakes (3.0 and 15.0 g, loaded in the extraction chamber) at 24.0 MPa and 35°C. CO_2 flow rate through the system was 0.5 L/min for both runs. When a higher amount of canola flakes was used, a consistent supply of oil onto the enzyme bed was maintained and therefore the total amount of oil contacting the enzyme bed was higher. In addition, a higher amount of water was received by the enzyme since more water would be extracted from a 15.0 g batch than from a 3.0 g batch. Such differences in the amount of oil and water introduced to the enzyme bed may be responsible for structural changes. However, micrographs of the enzymes under those conditions did not show any difference in appearance compared to that of an untreated enzyme (Fig. 4.7).

4.4. CONCLUSIONS

In the lipase-catalyzed hydrolysis of canola oil in $SCCO_2$, high pressure, CO_2 flow rate and oil content of $SCCO_2$ are parameters that apparently do not have any impact on the enzyme structure when observed under SEM. If there were any physical structural changes in the enzyme as a result of a change in these parameters, they could not be detected using an SEM at 50,000X magnification level. Changes at the molecular level cannot be observed by such a method.

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5. USING SUPERCRITICAL FLUID CHROMATOGRAPHY TO DETERMINE THE DIFFUSION COEFFICIENTS OF LIPIDS IN SUPERCRITICAL CO_2[1]

5.1. INTRODUCTION

Supercritical fluid chromatography (SFC), which was first introduced to analytical chemists for separation of mixtures in the supercritical state, has recently found applications in the determination of binary diffusion coefficients (D_{12}) of solutes using the Taylor-Aris peak-broadening technique. The D_{12} of solutes in SCFs play a major role in their extraction from the solid matrix as well as their dissolution from liquid mixtures for fractionation purposes. Also, in the enzyme-catalyzed reactions of materials in SCFs the D_{12} is an important parameter to determine how well a compound can migrate to the active site of enzyme as well as how well a product leaves the enzyme site to reach the bulk SCF.

Although earlier studies on the determination of D_{12} were carried out with custom-built systems, commercial SFC systems are now quite common. The principle for this technique is that when a pulse of a solute is introduced into a solvent stream flowing through a straight tube under laminar conditions, it broadens into a peak as a result of the combined effects of convection along the tube axis and molecular diffusion in the radial direction (Liong et al., 1991a). The solute peak generated under ideal conditions is Gaussian and the peak width can be correlated to the D_{12} of the solute in the solvent. Influence of pressure, temperature and addition of methanol on the D_{12} of acetone, benzene, naphthalene, 1,3,5-trimethylbenzene, phenanthrene,

[1] A version of this chapter was accepted for publication in the Journal of Supercritical Fluids.

pyrene and chrysene was investigated by Sassiat et al. (1987), using Taylor-Aris peak-broadening technique at 40-60°C and pressures up to 30.0 MPa in supercritical carbon dioxide ($SCCO_2$). Swaid and Schneider (1979) and Bueno et al. (1993) carried out similar studies in $SCCO_2$ at pressures and temperatures as high as 36.0 MPa and 60° C, respectively. Using dichloromethane, hexane and methanol as solvents, Lauer et al. (1983) investigated the effect of solvent on the diffusivity of benzene in $SCCO_2$.

The Taylor-Aris technique was also used in the determination of D_{12} of lipids in $SCCO_2$. Liong et al. (1991b; 1992) reported the D_{12} of several fatty acid methyl and ethyl esters at 35-45°C and 9.7-21.1 MPa in $SCCO_2$. Funazukuri et al. (1989; 1991; 1992) studied the D_{12} of several fatty acid methyl esters and some vitamins in $SCCO_2$ at temperatures up to 40°C and 16.0 and 25.0 MPa. The D_{12} of stearic and oleic acids at 40°C and pressures up to 16 MPa and that of linolenic acid at 41°C and pressures up to 18 MPa in $SCCO_2$ were reported by Dahmen et al. (1990). Catchpole and King (1994) determined the D_{12} of oleic acid and glycerol trioleate at 35°C and 20.1 and 25.1 MPa in $SCCO_2$.

Extraction of lipids from natural matrices using $SCCO_2$ is usually carried out at pressures higher than 25.0 MPa to achieve higher solubility levels. However, the literature lacks information on the D_{12} of fatty acids, their esters and other lipid components of interest at pressures >25.0 MPa. Thus, the objectives of this study were (a) to determine the D_{12} of several classes of lipids in $SCCO_2$ at pressures up to 36.0 MPa, and (b) to investigate the effect of pressure, temperature, presence of a secondary solvent/solute and the number and position of double bonds on the D_{12} of lipids.

5.2. MATERIALS AND METHODS

5.2.1. Materials

All lipid standards were purchased from Sigma Chemical Co. (Oakville, ON) with purities of ≥99%, except for trilinolenin and oleic acid ethyl ester, which were approximately 98% pure. Benzene (spectrophotometry/chromatography grade, OmniSolv) was obtained from EM Science (Gibbstown, NJ); n-hexane (HPLC grade) from Fisher Scientific (Nepean, ON); anhydrous ethanol, from Commercial Alcohols Inc. (Brampton, ON) and CO_2 (SFC/SFE grade) from Air Products (Allentown, PA).

5.2.2. SFC system and experimental design

An SFC system (SFC/GC Series 600, Dionex Canada, Mississauga, ON) was modified for D_{12} determination. The unit was equipped with a coiled (d_{coil}=16 cm) inert fused silica column (53 m x 100 μm I.D.) and a timed-split injector. A 0.075 sec injection time was applied in all experiments. The rotor in the injector was changed to one with 60 nL capacity to minimize injection volume. A UV/VIS detector (SFC/GC Series 600, Dionex Canada, Mississauga, ON) monitored the solute at applied wavelengths; 261 nm for benzene, 210 nm for oleic acid and 215 nm for all other lipids. The capillary column was inserted through the detector cell without any connections to ensure that the $SCCO_2$ flow was not disrupted. A narrow window opened on the outside surface of the fused silica tube, which was aligned with the slit of the UV detector cell, allowed the detection of solute inside the capillary column. A frit-restrictor (12 cm x 50 μm I.D.) attached to the end of the column and maintained at 125°C controlled the backpressure of the system as well as the $SCCO_2$ flow rate. Laminar flow of $SCCO_2$ at a velocity of 1.7-3.3 cm/s, depending on column pressure and temperature, was maintained. Once the modification of the SFC system was

completed, benzene was used to test the reliability of the system to reproduce literature values for its diffusivity.

The system was run for at least 2 hrs at the desired temperature and pressure prior to sample injection and the first run of each set was discarded. Pressure (25.0-36.0 MPa) and temperature (40-60°C) were kept constant during each set of experiments. An injection was made shortly after the peak from the previous injection was detected. Oleic, linoleic, linolenic and γ-linolenic acids, mono-, di- and triolein, trilinolein, trilinolenin and methyl and ethyl oleate were the lipid compounds studied. The samples were injected as is, without dissolving them in a solvent, except for the following cases where the secondary solvent effect was studied: 22% (w/w) oleic acid methyl ester in ethanol; 5, 10, 20, 50 and 75% (w/w) oleic acid in ethanol and 10, 20, 50 and 75% (w/w) oleic acid in hexane. At least triplicate injections of each sample were made and means±standard deviation were reported. The Dionex Al-450 Chromatography Automation Software Release 3.32 was used to collect and analyze the data.

5.2.3. Taylor-Aris peak broadening technique

Peak-width at half height was used to calculate D_{12} using the Taylor-Aris peak-broadening technique (Swaid and Schneider, 1979). Height equivalent to a theoretical plate, H, and D_{12} of the solute in supercritical fluid were determined using the following expressions, respectively (Swaid and Schneider, 1979):

$$H = \frac{L[w_{1/2}(t)]^2}{5.54 t_r^2} \qquad (5.1)$$

$$D_{12} = \frac{\overline{U}}{4}\left[H \pm \left(H^2 - \frac{r_0^2}{3} \right)^{1/2} \right] \qquad (5.2)$$

where L is the column length, $w_{1/2}(t)$ the peak-width at half height in time dimension, t_r the retention time, $\overline{U}$ the average velocity of the mobile phase and r_0 is the inside radius of the column. The negative root of equation (5.2) was used in all calculations since the positive root is for very low velocities; i.e. below the optimum velocity of $48^{0.5}D_{12}/r_0$ (Liong et al., 1991a).

Several factors can contribute to the error in this technique, as were discussed in detail by Levelt Sengers et al. (1993). Adsorption of solute onto the column wall can lead to peak tailing. Kirkland asymmetry factor, the ratio of the right half-width of the peak to the left half-width at 10% of the height (Bueno et al., 1993) was used to evaluate the symmetry of each peak as illustrated in Figure 5.1. To approach ideal Gaussian behavior, the asymmetry factor should be close to unity. The asymmetry factor was within the range of 0.99-1.10 for most samples of this study, but it was slightly higher for some samples as will be discussed later. Levelt Sengers et al. (1993) calculated skewness of the peaks to estimate their deviation from Gaussian. They reported skewness values of the order of 10^{-3}, while that of this study was at 10^{-10}-10^{-9} level.

The presence of centrifugal force acting on the solute molecules in a solvent stream flowing through a coiled tube is another source of error. Such secondary-flow effects can be neglected if the criteria given in equation (5.3) is met, thus resembling flow in a straight tube (Lauer et al., 1983; Liong et al., 1991b).

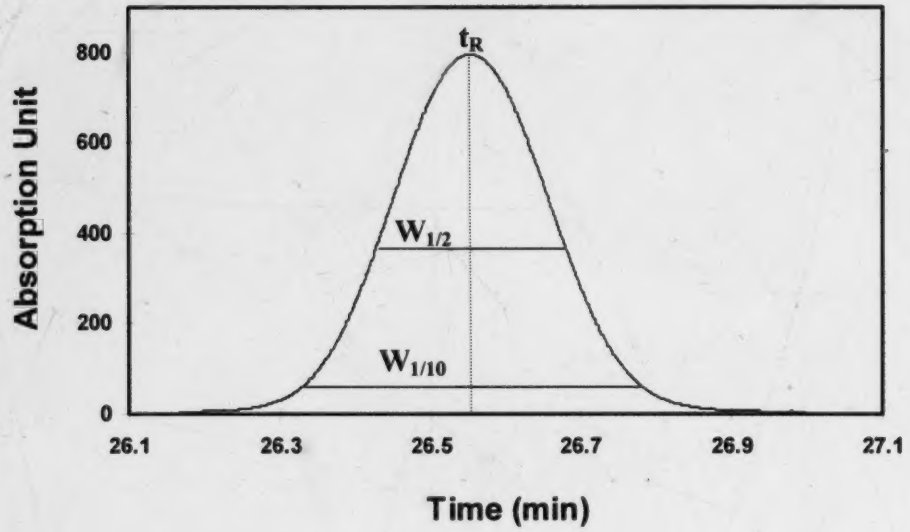

Figure 5.1. Typical chromatogram obtained in this study.

$$DeSc^{1/2} < 10 \qquad (5.3)$$

where

$$De = \frac{\rho u_0 d_{tube}}{\eta}\left(\frac{d_{tube}}{d_{coil}}\right)^{1/2} \qquad \text{(Dean Number)} \qquad (5.4)$$

and

$$Sc = \frac{\eta}{\rho D_{12}} \qquad \text{(Schmidt Number)} \qquad (5.5)$$

and ρ, η and u_0 are the density, viscosity and velocity of the supercritical fluid, and d_{tube} and d_{coil} are the diameters of the column and coil, respectively (Lauer et al., 1983). Density and viscosity data for CO_2 were taken from Angus et al. (1976) and Stephan and Lucas (1979) respectively. The above criteria were satisfied for all of the samples investigated in this study.

Another source of deviation is the presence of dead volume in the detection system. However, that was not the case in this study because of the modifications described for the detection system. The last possibility of error was from the signal-processing device. The Dionex software used to process the signal displayed data at time intervals of ±0.01 min. This level of accuracy was not sufficient to determine the peak-width for the narrow peaks obtained. Therefore, manual interpolation of computer data within the 0.01 min interval was carried out to determine the peak width at half-height with an accuracy level of ±0.0001 min for each peak. Such

computerized data acquisition is significantly better than the old systems relying on chart recorders.

5.3. RESULTS AND DISCUSSION

5.3.1. Diffusion coefficient of benzene

Experiments with benzene established the reliability of the SFC system in the pressure and temperature range of 25.0-35.0 MPa and 40-60°C, respectively (Fig. 5.2). Asymmetry factors for benzene were <1.05. The data were in good agreement with those reported by Bueno et al. (1993) where the D_{12} decreased with increasing CO_2 density. The effect of pressure and temperature on the D_{12} of benzene at 25.0-35.0 MPa and 40-60°C has been demonstrated in Figure 5.3. Similar to previous studies (Swaid and Schneider, 1979; Bueno et al., 1993; Levelt Sengers et al., 1993), D_{12} increased with temperature and decreased with pressure, following the changes in CO_2 density.

5.3.2. Effect of pressure, temperature, $SCCO_2$ density and secondary solvent on the diffusion coefficient of oleic acid

D_{12} of oleic acid was determined at 40, 50, 60°C and 25.0, 30.0, 36.0 MPa. Asymmetry factors of the peaks used for the diffusivity results reported were in general <1.1 except at the following conditions where they were slightly higher at <1.2: 25.0 MPa/40, 60°C, 30.0 MPa/60°C and 36.0 MPa/40°C. However, further evaluation of the results from 8 injections made at 36.0 MPa and 60°C showed that the D_{12} calculated using peaks with asymmetry factors up to 1.3 were similar. D_{12} values dropped with a further increase in the asymmetry factor up to 1.35 and those peaks were discarded.

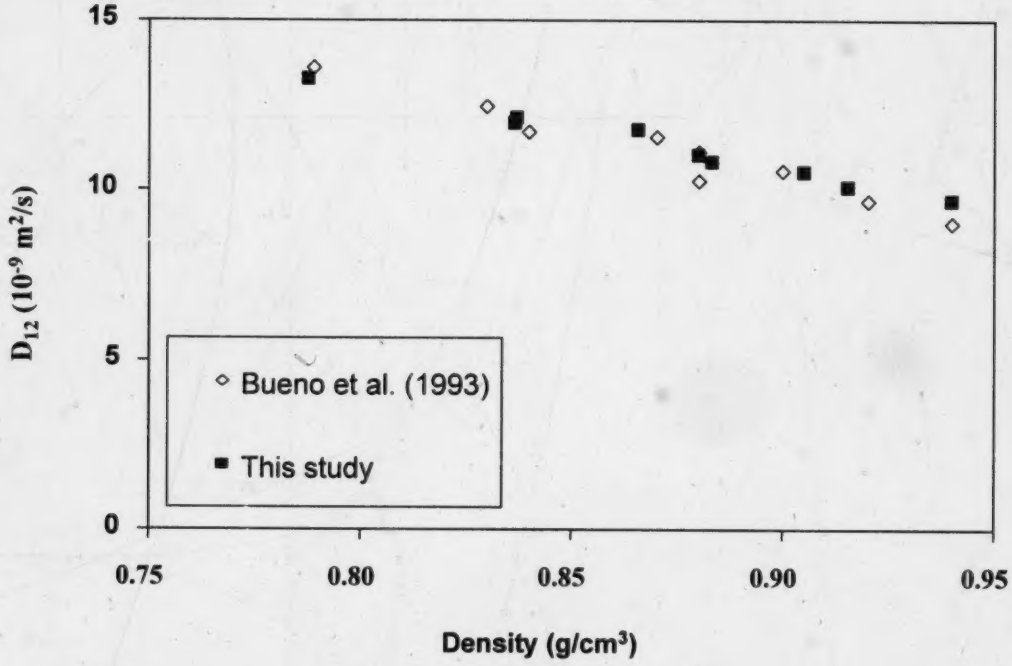

Figure 5.2. Diffusion coefficient of benzene in $SCCO_2$ as a function of CO_2 density.

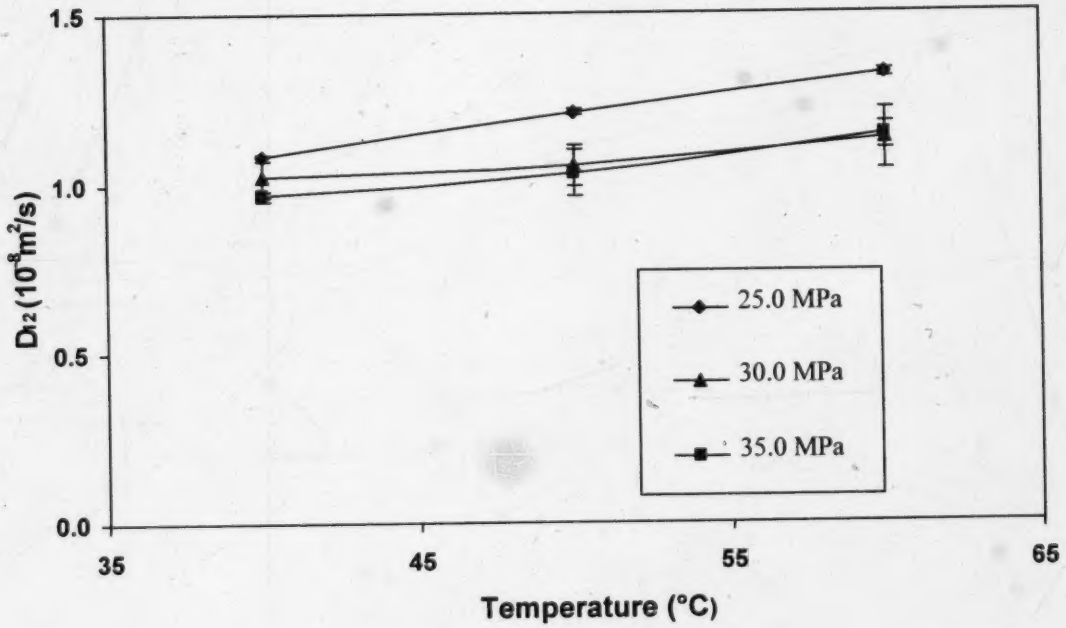

Figure 5.3. Effect of pressure and temperature on the diffusion coefficient (D_{12}) of benzene in $SCCO_2$.

The effect of pressure and temperature on the D_{12} of oleic acid is shown in Figure 5.4. As expected, D_{12} of oleic acid also increased with temperature and decreased with pressure; however, the effect of pressure was negligible in the 25.0-30.0 MPa range. Figure 5.5 depicts the decrease in the D_{12} of oleic acid with increasing $SCCO_2$ density, which is consistent with the results for benzene (Fig. 5.2). As the medium gets denser, collision frequency increases and the mean path of the molecules decreases. The results reported for naphthalene and stearic acid ethyl ester at high pressures indicate similar trends with pressure, temperature and density (Liong et al., 1991a; 1991b). The range of binary D_{12} of oleic acid determined in this study is somewhat smaller compared to that reported by Catchpole and King (1994) at 25.1 MPa and 35°C ($4.80\pm0.12\times10^{-9}$ m^2/s). This could be related to the difference in the methods of solute introduction in the two studies. Dahmen et al. (1990) reported a relatively higher value (1.08×10^{-8} m^2/s) for diffusivity of oleic acid at $SCCO_2$ density of 0.792 g/cm^3 at 16 MPa and 40°C, compared to the range of the results in this study. This difference could be due to the fact that they dissolved the solutes in ethanol prior to injection to $SCCO_2$. Dahmen et al. (1990) did not specify the concentration of oleic acid in ethanol used in their experiments but indicated that the presence of a secondary solvent would not affect the results. However, in this study the D_{12} of oleic acid in $SCCO_2$ was considerably higher when dissolved in ethanol at a concentration less than 50% (w/w) (Fig. 5.6). Such a concentration effect was not observed in hexane. The molecular interactions that lead to an apparent enhancement in the diffusivity of oleic acid at a relatively higher concentration of a polar secondary solvent such as ethanol require further investigation, since ethanol is a popular co-solvent used when extracting lipids from various natural matrices with $SCCO_2$. Because the lipid solute

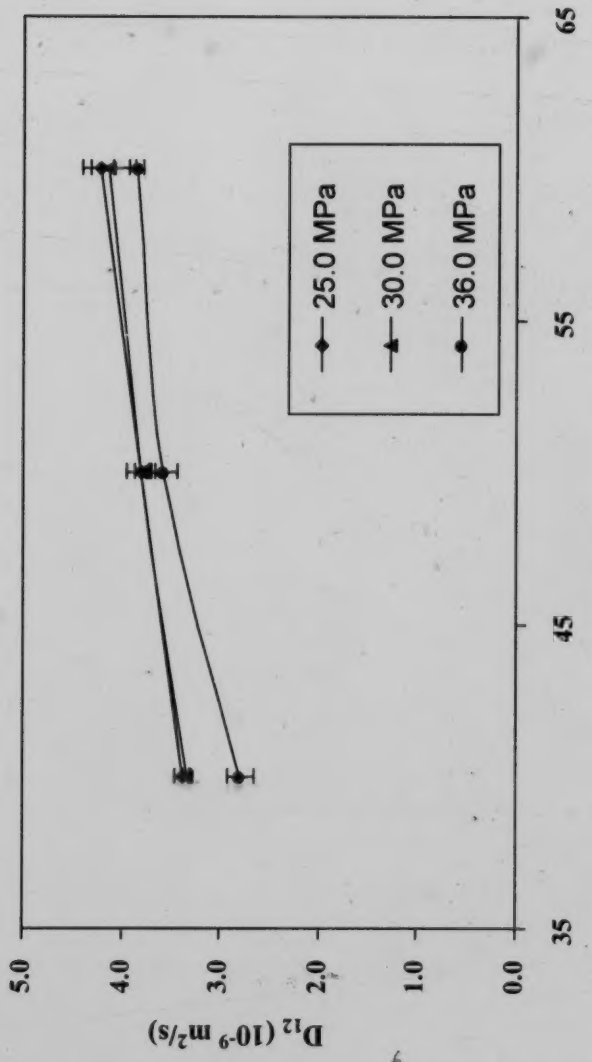

Figure 5.4. Effect of pressure and temperature on the diffusion coefficient (D_{12}) of oleic acid in $SCCO_2$.

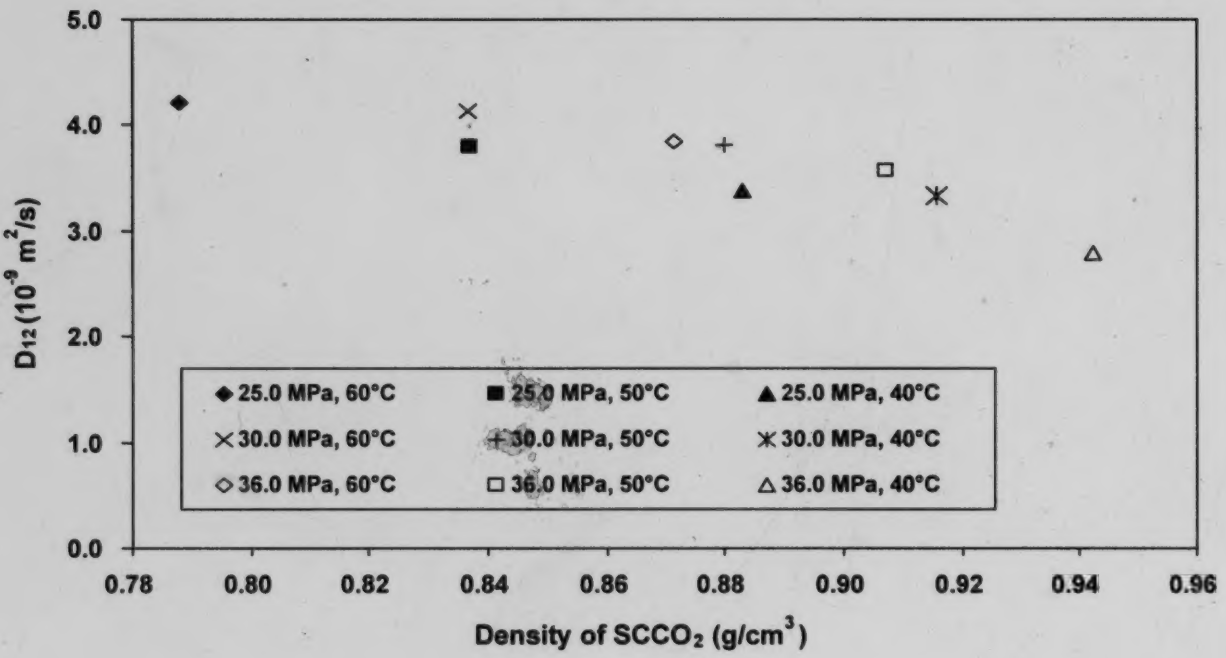

Figure 5.5. Diffusion coefficient of (D_{12}) of oleic acid as a function of $SCCO_2$ density.

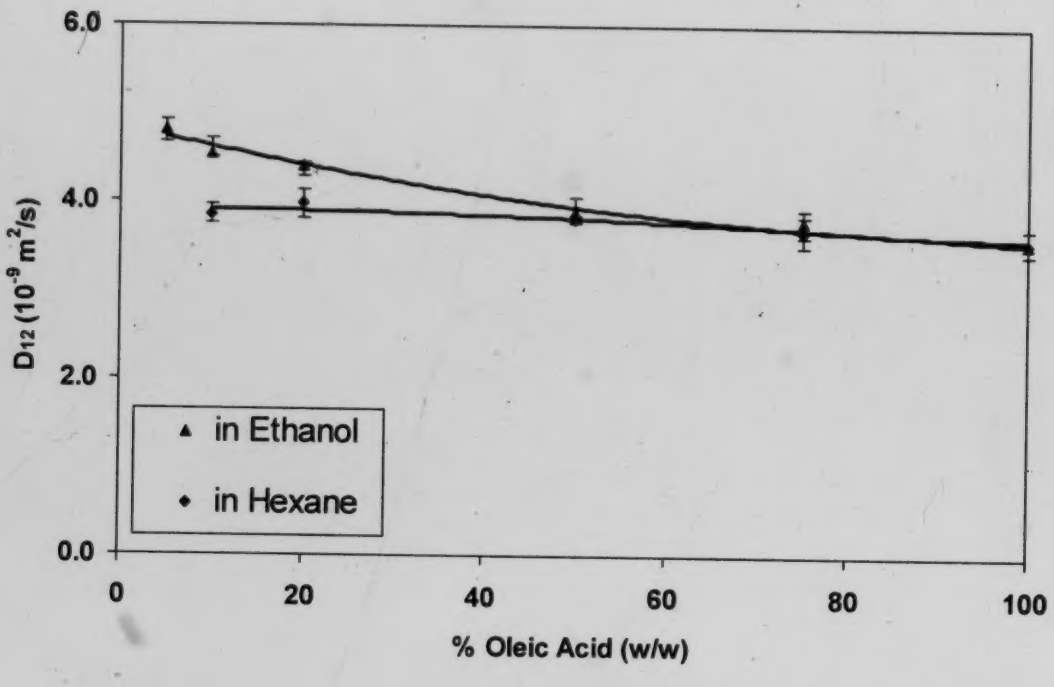

Figure 5.6. Effect of secondary solvent on the diffusion coefficient (D_{12}) of oleic acid in $SCCO_2$ at 36.0 MPa and 50°C.

and the secondary solvent, ethanol, were both present in trace amounts in $SCCO_2$, further evaluation should take the coupled effect from the secondary solvent into account (Least, 1990).

In Figure 5.7, D_{12} of an 11% (w/w) oleic acid solution in ethanol is compared to that of a pure sample over a pressure and temperature range of 25.0-36.0 MPa and 40-60°C, respectively. The apparent increase in the D_{12} is due to the presence of the secondary solvent, ethanol. In addition, the effect of ethanol dominated any pressure effect since the different isobars overlapped, whereas the decreasing effect of pressure (30.0-36.0 MPa) was apparent for the pure sample. A similar behavior was observed when working with oleic acid methyl ester, where the D_{12} of a 22% (w/w) solution of oleic acid methyl ester in ethanol shifted from that of a pure sample at 36.0 MPa and 50°C, $4.97\pm0.06\times10^{-9}m^2/s$ vs. $4.65\pm0.08\times10^{-9}m^2/s$, respectively. As expected, this result is lower than $6.59\pm0.05\times10^{-9}m^2/s$ reported by Funazukuri et al. (1991) for the D_{12} of oleic acid methyl ester at 16.0 MPa and 40°C. When working with naphthalene, benzene and linoleic acid methyl ester, Funazukuri et al. (1989) reported little or no effect of n-hexane as a secondary solvent. This may be true for certain solutes and solvents over a limited range of pressure, temperature and concentration, but apparently it is not the case for all solutes/solvents at all conditions. Lauer et al. (1983) compared the D_{12} of benzene in $SCCO_2$ when it was dissolved in different organic solvents. They observed little effect from the secondary solvent and assumed that it would be the same for caffeine and naphthalene. Feist and Schneider (1982), Liong et al. (1991a; 1991b) and Catchpole and King (1994) dissolved the solutes in $SCCO_2$ prior to injection to the column. Obviously, this method is the best approach to introduce the solute into the column since the problems associated with the injection of

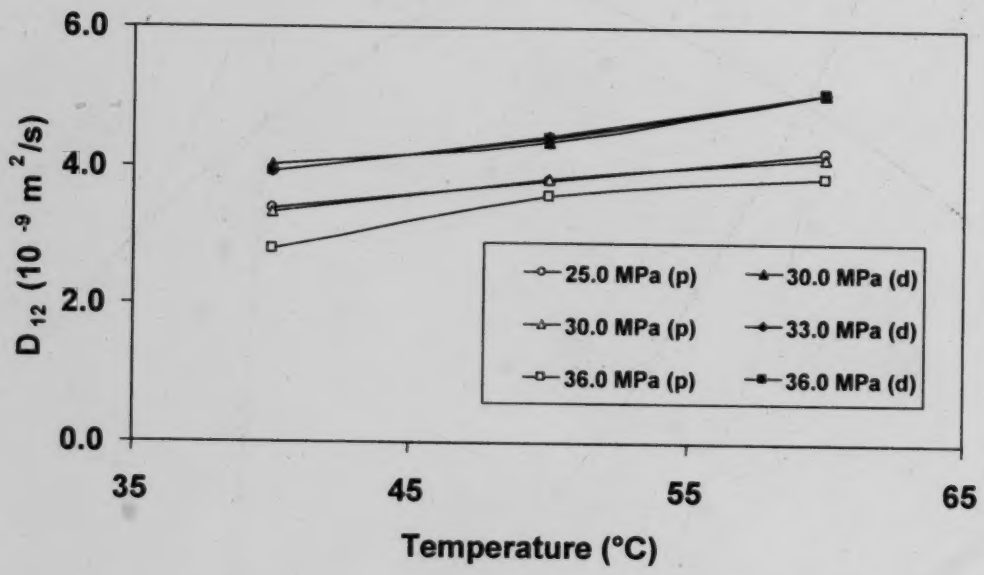

Figure 5.7. Effect of secondary solvent on the diffusion coefficient of (D_{12}) of oleic acid in $SCCO_2$ at different pressures and temperatures. [p=pure oleic acid, d=11% (w/w) oleic acid in ethanol].

solutes of low solubility or solid solutes into the high pressure flow of supercritical fluid are eliminated and no effect from a secondary solvent is introduced into the results. This approach, however, requires another high pressure loop to be added to the system for the solubilization of the solute in $SCCO_2$, which is not practical with commercial SFC units.

5.3.3. Diffusion coefficients of various lipids

The D_{12} of various pure lipid components at 50°C and 36.0 MPa along with the corresponding asymmetry factors for the peaks used in these determinations are reported in Table 5.1. Except for the peaks of trilinolein and trilinolenin, for which the asymmetry factors are 1.3 and 1.2, respectively, all asymmetry factors are <1.1. Funazukuri et al. (1992) have also reported asymmetry factors as high as 1.3.

Table 5.1. Diffusion coefficient (D_{12}) of pure lipids (mean±standard deviation) in $SCCO_2$ determined at 50°C and 36.0 MPa and the asymmetry factors of their corresponding peaks.

Compound	D_{12} ($10^{-9}m^2/s$)	Asymmetry Factor
Oleic Acid	3.57±0.18	<1.10
Linoleic Acid	3.33±0.35	<1.02
Linolenic Acid	2.02±0.17	<1.01
γ-Linolenic Acid	2.56±0.12	<1.09
Methyl-Oleate	4.65±0.08	<1.07
Ethyl-Oleate	4.17±0.05	<1.05
Dilinolein	2.22±0.04	<1.10
Trilinolein	2.03 ±0.12	<1.30
Trilinolenin	2.23±0.04	<1.20

A decrease in the D_{12} of oleic, linoleic and linolenic acids was observed in $SCCO_2$ at 36.0 MPa and 50°C (Table 5.1) with an increase in the number of double bonds, i.e. degree of unsaturation. In a similar study on stearic, oleic and linolenic acids, Dahmen et al. (1990) observed little or no difference in the D_{12} of stearic and oleic acids at 40°C and 12-16 MPa. However, there was a significant drop in the D_{12} from oleic to linolenic acid. Although they dissolved these fatty acids in ethanol, such a drop in the D_{12} is consistent with our results and can be correlated with the structural changes among these fatty acids. The D_{12} of γ-linolenic acid (6,9,12-octadecatrienoic acid, ω-6) was determined at 36.0 MPa and 50°C and compared with that of α-linolenic acid (9,12,15-octadecatrienoic acid, ω-3) as shown in Table 5.1. In this case, the repositioning of a double bond resulted in a change in the D_{12}. γ-Linolenic acid, which is spatially more symmetric, demonstrated a higher D_{12} than α-linolenic acid, a less symmetric compound structurally.

To study the alkyl effect, the D_{12} of methyl and ethyl oleate were determined at 36.0 MPa and 50°C (Table 5.1). Diffusivity of esters was higher than that of free fatty acids. This may be attributed to structural differences and possible molecular association of the fatty acid. Also, a higher value of D_{12} was observed for methyl oleate compared to that of ethyl oleate indicating an alkyl effect. This is consistent with the data for docosahexaenoic acid ($C_{22:6}$) methyl and ethyl esters at 35-45°C and 9.7-18.8 MPa reported by Liong et al. (1991b, 1992). The D_{12} of methyl oleate obtained in this study compared well with that reported by Funazukuri at al. (1991) at 40°C and 16.0 MPa ($6.59\pm0.05\times10^{-9}$ m^2/s).

The D_{12} of dilinolein, trilinolein and trilinolenin were measured at 36.0 MPa and 50°C (Table 5.1). A value of $3.55\pm0.37\times10^{-9}$ m^2/s has been reported by Catchpole

and King (1994) for triolein at 25.1 MPa and 35°C. Triglyceride diffusivity was lower than that of its corresponding free fatty acid for trilinolein but higher for trilinolenin. The differences in their molecular weight and size as well as the degree of unsaturation and spatial configuration are important factors. In addition, more polar nature of dilinolein compared to trilinolein should affect molecular interactions.

Determination of the D_{12} of monolinolein and triolein was also attempted to complete the series of the lipids given in Table 5.1. However, it was not successful due to extensive tailing and high asymmetry factors of 1.4 and 2.2 for triolein and monolinolein, respectively. Adsorption on the column wall and time-split injector due to the viscous nature of these samples is probably responsible for this problem. Even though not very reliable, D_{12} based on $w_{1/2}$ obtained by doubling the width of left half of the peak at half-height were determined for these compounds since tailing was only apparent in the lower half of the right side of the peak. Such an approach resulted in D_{12} of $1.91\pm0.09\times10^{-9}$ and $2.09\pm0.07\times10^{-9}$ m^2/s for triolein and monolinolein, respectively, which are comparable to those of other lipids in the same class (Table 5.1). However, a loop at supercritical conditions to solubilize the sample in $SCCO_2$ prior to injection is recommended.

In an effort to study the behavior of a binary mixture of lipids in $SCCO_2$, a 1:3 (w/w) mixture (~50/50, mole%) of oleic acid and triolein was injected into the SFC system. Only one peak was detected, which resulted in a D_{12} similar to that of triolein alone, $2.26\pm0.22\times10^{-9}$ m^2/s vs. $1.91\pm0.09\times10^{-9}$ m^2/s, respectively, based on the corrections for the $w_{1/2}$. Likewise, the asymmetry factor was <2.2. Oleic acid with a D_{12} of $3.57\pm0.18\times10^{-9}$ m^2/s did not seem to contribute to the overall D_{12} at the selected concentration level. However, since both oleic acid and triolein have UV absorbance,

what is detected in the detector is a combination of the absorbances of both compounds. Selective detection of the solutes would have separated the diffusion behavior of the compounds from each other. Despite the high asymmetry factor for the oleic acid-triolein mixture, it seems that the presence of a free fatty acid along with a triglyceride with the same fatty acid moiety will result in an apparent D_{12} for the mixture very close to that of the triglyceride. Whether this result is valid for systems containing other triglycerides and their fatty acids and over a wide range of concentrations needs to be examined. Developing a correlation technique for multi-component mixtures and predicting the diffusion behavior of complicated mixtures such as fats and oils in $SCCO_2$ will require further investigation.

5.4. CONCLUSIONS

This study demonstrated that the operating pressure and temperature, concentration of secondary solvent and position and number of double bonds are important parameters in the determination of binary D_{12} of lipids in $SCCO_2$. The D_{12} of oleic acid was reduced with pressure and increased with temperature, leading to a decrease with $SCCO_2$ density. The presence of ethanol as a secondary solvent at a concentration above 50% (w/w) resulted in an increase in the D_{12} of oleic acid and oleic acid methyl ester, D_{12} for various lipid classes decreased according to the following order: fatty acid ester > fatty acid > triglyceride. Determination of D_{12} of various lipids at 36.0 MPa in this study significantly increased the pressure maximum for such data available in the literature. More studies are needed for better understanding of diffusion behavior of complex lipid mixtures during supercritical processes.

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6. CONCLUSIONS AND RECOMMENDATIONS

The study of extractions and reactions in supercritical fluids is growing rapidly and supercritical fluid (SCF) technology is slowly replacing some of the traditional methods of extractions and reactions using organic solvents. The parameters affecting extractions and reactions in SCFs with a specific emphasis on the enzymatic reactions in supercritical CO_2 ($SCCO_2$) were discussed in chapter 1.

Using SCFs as media for reactions solves some of the problems associated with lower boiling points of the conventional solvents since the operation is at higher pressures and temperatures (Tiegs, 1996). The rate and productivity of reactions in a supercritical environment are very sensitive to physical parameters such as pressure, temperature and composition. While maintaining a high reaction rate, the selectivity can be controlled and the separation of the products can then be carried out by utilizing the phase behavior of the SCF (Lee et al., 1993) and the products. Even though SCFs have been studied quite extensively, there is still a need for further investigations to improve the understanding of the complex phenomena happening in such media. CO_2 as a supercritical solvent offers advantages like low price, availability, safety, ease of recovery of the solute and moderate critical point. Thus, it has been a favorite non-aqueous solvent in which to study enzymatic reactions. However, extensive investigation of a given enzyme in a somewhat broad range of pressure, temperature and other parameters has not been carried out to allow determination of optimum conditions. Despite the fact that it has been quite useful in many applications, including the food industry, there have been applications in which $SCCO_2$ has not been considered a preferred solvent. For example, Carvalho et al. (1995) reported a direct negative CO_2 effect in the subtilisin-catalyzed

transesterification of benzyl alcohol with vinyl butyrate. Propane as an alternate SCF provided higher maximum activity than CO_2. Similar adverse effects have also been observed by Nakamura et al. (1990) and Kamat et al. (1992). Although $SCCO_2$ treatment with microbubbles in aqueous solution (Balaban et al., 1991; Ishikawa et al., 1996), pressurization-depressurization (Aaltonen and Rantakylä, 1991; Randolph et al., 1991; Lozano et al., 1996; Cano et al., 1997), and treatment at different levels of pressure, temperature, moisture content and exposure time (Dunford and Temelli, 1996) have been shown to inactivate enzymes with undesirable activities, the proper use of enzymes in nonaqueous environments has not caused significant enzyme inactivation for several days in most studies.

There are different interpretations about the negative effect of high pressure on enzymatic reactions. Although the finding of Kamat et al. (1992; 1995) that CO_2 bonds covalently to the enzyme is a relatively new piece of information regarding the enzyme behavior in $SCCO_2$ medium, it cannot yet explain the negative effect observed when working with other SCFs such as ethane. As well, the covalent bonding of CO_2 does not at all mean that enzyme inactivation is involved. In fact, Lozano et al. (1996) have shown that the stability of α-chymotrypsin has increased with pressure, although they observed a decrease in the enzyme activity with an increase in the temperature from 25°C to 40 and 60°C. Therefore, while the application of SCFs with lower critical temperatures isneeded, employing a higher pressure does not produce any problem as long as the phase behavior of the system is respected. Phase behavior of a system affects the progress of the reaction since phase behavior dictates the partitioning of the reaction components between the phases involved. Partial molar volumes and calculated activation volumes determine if higher pressures favor the reaction.

The driving force of an enzymatic reaction can be maintained if it is carried out in a continuous manner. The advantage of a continuous system over a batch system other than having a consistent flow is continuous withdrawal of the products which may prohibit the propagation of the thermodynamic equilibrium towards higher production. Accumulation of some products such as free fatty acids (FFA) can inhibit enzyme activity (Lencki et al., 1998).

The application of enzymatic reactions in SCFs to food industry exhibits great potential. The transesterification of cocoa butter substitutes, for example, can take place only in media where the water content is extremely low, such as SCFs (Saito, 1995). This will suppress the formation of mono- and diglycerides (MG and DG, respectively) which are produced by hydrolytic reactions (Saito, 1995). The production of specific fatty acid esters similar to the flavorings used in margarine, imitation dairy products, confections and other prepared foods is possible with enzymatic esterification in SCFs. As well, monoglycerides, which are widely used as emulsifiers in food industry, can be prepared.

SCFs can be used as solvents in the extraction of heat labile compounds at low temperatures (Stahl et al., 1988). In order to manipulate the extraction and reaction rate as well as their selectivity, the main parameters to control are pressure, temperature and feed composition. In a SCF, the separation of the reaction products can be carried out by utilizing the phase behavior of the SCF (Lee et al., 1993) and the products. The analysis of the separated products can then be followed using a supercritical fluid chromatography (SFC) system. Currently, commercial pilot-plant and industrial scale supercritical fluid extractors and separators are available. SFC systems connected on-line to supercritical fluid extraction, reaction or separation units will become available in the near future.

The applicability of SCFs to the fast growing enzyme technology has provided an alternative to the use of organic solvents to conduct enzymatic reactions. Despite the availability of numerous SCFs, $SCCO_2$ is still the most commonly used solvent for food applications in research and industry. This is mainly due to its affordability, safety and inertness.

With the latest developments in SFE technology and its growing involvement in many processes including those in the flavors and spice industry, it is quite appropriate to incorporate such a technique to extract fats and oils directly from their sources and convert them to higher value products through an on-line enzymatic reaction. Developing such an on-line connection between the extractor and the reactor will simplify conventional processing steps and as a result will save time, energy and other resources.

The lipase-catalyzed hydrolysis of canola oil in $SCCO_2$ was performed at different pressures and temperatures using a continuous reactor system (chapter 2). The immobilized lipase, Lipozyme IM, lost 34% of its activity (based on the drop in TG disappearance) at 38 MPa and 55°C over a 24 h reaction period. The production of MG, DG and glycerol was affected by a change in pressure and temperature over 10-38 MPa and 35-55°C, respectively. At 24 MPa and 35°C, the enzyme showed the highest performance and therefore, more production of FFA was achieved. Because of the poor solubility of oil at the lowest pressure of this study (10 MPa), it resulted in the lowest FFA production. The highest conversion rate as well as the highest MG and DG production was obtained at 24-38 MPa and 35-55°C, which was 65-70% in a 4 h continuous run.

In the hydrolysis of canola oil in $SCCO_2$, the product composition was affected by a change in the CO_2 flow rate. The extent of hydrolysis of canola oil was improved by decreasing the flow rate of CO_2.

The conventional hydrolysis of oil involves a high pressure (>250°C), which is destructive to polyunsaturated fatty acids. Furthermore, such method is very labor- and energy-intensive (Linfield et al., 1984). As well, enzymatic reactions in organic solvents are suffering from a low reaction rate so that their industrial applications are not appropriate at this time. The ever-growing SCF technology is going to compete with traditional enzyme-catalyzed reactions in aqueous media as well as those performed in organic solvents. With such developments in both extraction and reaction processes, the convenience of on-line reaction of the extracted solutes will merge these two technologies to avoid several unnecessary steps involved in the independent extraction and reaction processes. On-line extraction-reaction of canola oil in $SCCO_2$ was performed successfully and results indicated different product patterns due to a change in the quantity of canola flakes in the extractor, CO_2 flow rate and enzyme load (chapter 3). Selective modification of any of these parameters can improve the content of a given species in the reaction product.

The DG concentration in the product was enhanced with an increase in canola load in the extraction chamber. The pattern in the introduction of oil to the reactor changed more dramatically when a smaller amount of canola flakes was used. For example, an increase in the oil load resulted in a lower production of FFA at the early stages of the run. However, towards the end of the run where the supply of oil to the enzyme was limited, FFA concentration in the product increased. A decrease in the quantity of the introduced oil promoted the full hydrolysis of oil and while there was still some TG in the product samples, intermediate products were not formed. It

is noteworthy that the presence of lipoxygenase in the canola flakes did not interfere with the extraction and reaction of this study since lipoxygenase would require oxygen to oxidize fatty acids and oxygen was not available in the CO_2 environment.

Although, it was expected that an increase in the enzyme load in the reaction cell would improve the production level, the extra resistance to the flow of oil-laden $SCCO_2$ reversed the results. However, the FFA content was increased. To take full advantage of the improvements without compromising the production rate of the reaction certain scale-up parameters such as an increase in the dimensions of the extraction and reaction chambers have to be considered.

Lipase-catalyzed hydrolysis of canola oil in $SCCO_2$ show potential for conversion of fats and oils to higher value products such as MG, DG, FFA and glycerol. MG and DG are primarily used as emulsifiers in food applications. Glycerol and different FFA are used in the production of such products as detergents, surfactants, cosmetics and health related chemicals. MG and DG are traditionally produced by glycerolysis reaction which is carried out at a very high temperature (250°C) resulting in a dark-colored product (Temelli et al., 1996). Furthermore, the separation of the products from the FFA and their metallic salts in the traditional method involves a distillation step to achieve a higher MG concentration of ~90%. This step introduces flavors and odors associated with the presence of FFA and their metallic salts in the product (Temelli et al., 1996). Glycerol, which is a compound of many diverse uses, is obtained from the hydrolysis of fats and oils.

Although canola flakes were used in the on-line extraction-reaction process in this study, cold-press cake can also be used as the starting material. Such consideration is appealing for both traditional extraction plants and the new on-line extraction-reaction process. Press cake contains about 20% (w/w) oil (Stahl et al.,

1988) and it can be a good raw material for the on-line extraction-reaction process using $SCCO_2$. Thus, organic solvent extraction and the extra steps involved with the organic solvent removal can be eliminated. Furthermore, meal desolventization will not be necessary and the meal left from the SFE/SFR process will not have any safety issues as it is used as animal feed. At the same time the cold-press oil and $SCCO_2$ extracted oils can keep their high market value since no organic solvent is involved in processing.

Although for the sake of understanding the phenomena occurring in the extraction-reaction processes, a lower CO_2 flow rate was applied in this study, a higher CO_2 flow rate can be used in industrial-scale extraction of oil in order to speed up the process. For example, decaffeination of coffee beans are carried out in an industrial scale in Houston, Texas, where 6800 kg of beans are extracted every 30-60 min (McHugh and Krukonis, 1994).

Possible microstructural changes in the immobilized lipase from *Mucor miehei*, Lipozyme IM, were investigated using Scanning Electron Microscope (SEM) analysis (chapter 4). Although free enzymes may undergo severe structural changes when they are exposed to high pressures and excessive water content, immobilized lipase from *Mucor miehei* did not undergo any structural changes that could be observed by SEM after being treated with different levels of pressure, CO_2 flow rate and oil content. When enzymes are immobilized, they are bound to a solid matrix, which can protect enzyme structure at different conditions. To properly observe the enzymes using SEM, a higher magnification is required for smaller enzymes. The electron microscope images indicated a size range of 35-55 nm for spheres observed on the anionic exchange resin which were interpreted to be group of *Mucor miehei* lipase. If there are any physical structural changes in the enzyme at the molecular

level as a result of a change in the extraction-reaction parameters, they could not be detected using a SEM at 50,000X magnification level. Therefore, to identify such possible deformations at the molecular level, another suitable method such as Infrared or Mass spectrometry should be applied.

To explain various phenomena taking place during extraction, reaction and separation processes and to predict the diffusion rates of solutes in SCFs, knowledge of the binary diffusion coefficient of the components involved in such processes is essential. This parameter was measured (chapter 5) for several lipid classes including TG, FFA, DG and fatty acid esters using Taylor-Aris peak-broadening technique at different conditions.

The operating pressure and temperature, concentration of secondary solvent and position and number of double bonds were important parameters affecting the binary diffusion coefficients of lipids in $SCCO_2$. Diffusion coefficient of oleic acid decreased with a reduction in $SCCO_2$ density. An increase in the diffusion coefficient of oleic acid and oleic acid methyl ester was observed by an increase in the concentration of ethanol as a secondary solvent to >50% (w/w), which may be attributed to the simultaneous detection of both species in the UV detector as well as intermolecular interactions. The following order was observed in the diffusion coefficients of various lipid classes: fatty acid ester > fatty acid > triglyceride. Determination of diffusion coefficients of various lipids at 36.0 MPa in this study extended the pressure maximum for such data available in the literature. Similar studies on complex lipid mixtures are needed to develop a method to estimate the diffusion behavior of the components of crude samples during supercritical processes. Selective detection of the mixture components can reduce the problems associated with the interactions occurring during such measurements.

There is great potential for developing new food processes using sequential extraction/reaction/fractionation schemes. Ingredient industry, especially fats, oils and flavors, are going to benefit from such on-line operations. The on-line extraction-reaction process investigated in this study is a step forward in such process development. However, more work is needed for optimization of the process.

The high cost of enzyme is a big concern when enzyme activity is lost in reaction. Therefore, pressure, temperature and water content have to be optimized to maximize and maintin enzyme activity. Furthermore, different types of enzymes have to be examined to find a proper enzyme for a specific application. Physical properties of SCFs need to be properly correlated with the activity, specificity and stability of the enzymes. Different parameters affecting enzyme activity and those affecting the physical properties of the supercritical solvent must be independently studied and every reaction (hydrolysis, esterification etc.) for a given enzyme or a group of enzymes need to be investigated separately. A database which can allow the appropriate comparison of the effect of different parameters on a certain system is crucial since it can lead to a better understanding of the effects and their interactions. Some enzymes such as glucoamylase, galactosidase, glucose oxidase, glucose isomerase, alcohol dehydrogenase and catalase need to be considered for conducting enzymatic reactions in SCFs. Also, biotechnological advancements such as genetic engineering work are necessary to produce enzymes with better tolerance against parameters that may be destructive to enzymes. There is not enough information to support a direct negative pressure effect on the enzyme activity. But, inability to withstand conditions such as high temperature and extra water are among those that enzymes suffer from. With the enzymes currently available, more research is needed

to focus on parameters such as water content, enzyme dispersion methods and enzyme-bed geometry, etc.

Selective esterification of glycerol with oleic acid by immobilization and complexation of glycerol by Castillo et al. (1994) was a successful application. This approach can be extended to other reactions as well using such complexing agents as phenylboronic acid since they appear to be very helpful in limiting unnecessary by-products. Controlling the water content of enzymatic reactions is crucial for better control on product composition. Using salt hydrate as a source of water can improve the selectivity and specificity of the reactions. More attention towards the application of this approach in food industry is needed.

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